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GLYCOGEN DEBRANCHING ENZYME ACTIVITY IN THE MUSCLES OF MEAT PRODUCING ANIMALS

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To my parents, and
to the memory of Utu

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ABSTRACT

Muscle glycogen exists in two forms: low molecular weight pro-glycogen and high molecular weight macro-glycogen. The degradation of glycogen to glucose-1-phosphate and free glucose is catalysed by glycogen phosphorylase together with glycogen debranching enzyme (GDE). The process in which glycogen is broken down via anaerobic pathways to lactate, results in the acidification of the muscles and has a great influence on meat quality. Thus, the overall aim of this thesis was to characterise the *post mortem* action of GDE in muscles of meat production animals (pigs, cattle and chickens). Interest was focused on the differences in GDE activity between fast twitch glycolytic muscles and slow twitch oxidative muscles. The effects of pH, temperature, RN genotype (PRKAG3 gene), and of time *post mortem* on GDE activity were also investigated.

This thesis showed that there are differences in GDE activity between animal species and between different muscles of an animal. It was shown that in pigs and cattle, higher GDE activity and phosphorylase activity exists in the fast twitch glycolytic muscles than in slow twitch oxidative muscles of the same animal. Thus, the high activity of these enzymes enables a faster rate of glycogenolysis in glycolytic *M. longissimus dorsi* compared to oxidative *M. masseter*. In chicken muscles, the GDE activity was low compared to pig or cattle muscles. Furthermore, the GDE activity in the glycolytic *M. pectoralis superficialis* was lower than in more oxidative *M. quadriceps femoris* despite the high phosphorylase activity in the former. The relative ratios between phosphorylase and GDE activity were higher in fast twitch glycolytic muscles than in slow twitch oxidative muscles of all studied animals. This suggests that the relatively low GDE activity compared to the phosphorylase activity in fast twitch glycolytic muscles may be a protection mechanism in living muscle against a very fast pH decrease.

Chilling significantly decreased GDE activity and below 15 °C porcine GDE was almost inactive. The effect of pH on GDE activity was only minor at the range normally found in *post mortem* muscles (pH 7.4 to 5.0). The GDE activity remained level for several hours after slaughter. During the first hours *post mortem*, GDE activity was similar in RN^- carrier pigs and in wild type pigs. However, the GDE activity declined faster in *M. longissimus dorsi* from wild type pigs than in the RN carrier pigs, the difference between genotypes was significant after 24 h *post mortem*. Pro-glycogen and macro-glycogen contents were higher, pH decrease was faster and ultimate pH was lower in RN^- carrier pigs than in wild type pigs. In the RN^- carriers, the prolonged high GDE activity level may enable an extended pH decrease and lower ultimate pH in their muscles.

In conclusion, GDE is not the main factor determining the rate or the extent of *post mortem* glycogenolysis, but under certain conditions, such as in very fast chilling, the inhibition of GDE activity in meat may reduce the rate of pH decrease and result in higher ultimate pH. The rate and extent of pH decrease affects several meat quality traits.

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Helsinki, September 2006

Maria Ylä-Ajos

CONTENTS

ABBREVIATIONS

abs	absorbance
acetyl-CoA	acetyl coenzyme A
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
ATP	adenosine triphosphate
ATPase	ATP hydrolase
B_7	6^3-α-maltotriosylmaltotetrose
cAMP	cyclic 3,5-adenosine monophosphate
CV	coefficient of variation
DFD	dark, firm and dry meat
fast B_5	6^3-α-glucosylmaltotetrose
FG	fast twitch, glycolytic muscle
FOG	fast twitch, oxidative, glycolytic muscle
GDE	glycogen debranching enzyme
glucosidase	amylo-1,6-glucosidase (dextrin 6-α-glucohydrolase, EC 3.2.1.33)
GP	glycolytic potential
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
H^+	proton
IMP	inosine monophosphate
ITP	inosine triphosphate
LDH	lactate dehydrogenase
limit dextrin	phosphorylase limit dextrin of glycogen
MG	macro-glycogen
M.W.	molecule weight
n	number of samples
NAD^+	nicotinamide dinucleotide (oxidized form)
NADH	nicotinamide dinucleotide (reduced form)
n.s.	statistically non significant
PCA	perchloric acid
PCr	phosphocreatine
PG	pro-glycogen
P_i	inorganic phosphate
pH_1	pH value measured 35 min after stunning in cattle and pig and at 25 min after stunning in poultry
phosphorylase, PHOS	glycogen phosphorylase
pH_u	ultimate pH, measured at 24 h *post mortem* in poultry and pig and at 48 h *post mortem* in cattle
PSE	pale, soft and exudative meat

RN^-	A dominant allele of PRKAG3 gene affecting unfavourably on pig meat quality
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
s.e.	standard error
SO	slow twitch, oxidative muscle
transferase	maltooligosaccharide transferase (1,4-α-glucan:1,4-α-glucan 4-α-glycosyltransferase, EC 2.4.1.25)
UDPG	uridine diphosphate-glucose
UTP	uridine triphosphate
w.w.	wet weight

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by Roman numerals I-IV.

I Kylä-Puhju, M., Ruusunen, M. & Puolanne, E. (2005). Activity of porcine muscle glycogen debranching enzyme in relation to pH and temperature. Meat Science, 69(1), 143-149.

II Ylä-Ajos, M., Ruusunen, M. & Puolanne, E. (2006). The significance of the activity of glycogen debranching enzyme in glycolysis in porcine and bovine muscles. Meat Science, 72(3), 532-538.

III Ylä-Ajos, M., Ruusunen, M. & Puolanne, E. (2006). Glycogen debranching enzyme and some other factors relating to *post mortem* pH decrease in poultry muscles. Journal of the Science of Food and Agriculture. In press.

IV Ylä-Ajos, M.S., Lindahl, G., Young, J.F., Theil, P.K., Puolanne E., Enfält A.-C., Andersen, H.J., Oksbjerg, N. (2006). Post-mortem activity of the glycogen debranching enzyme and change in the glycogen pools in porcine M. longissimus dorsi from carriers and non-carriers of the RN^- gene. Meat Science. In press.

RESEARCH INPUT AND AUTHORSHIP OF ARTICLES (I-IV)

This thesis is a summary of the research reported in the four (I to IV) appended papers. The research input and authorship of the papers are as follows:

I *Kylä-Puhju, M., Ruusunen, M. & Puolanne, E. (2005). Activity of porcine muscle glycogen debranching enzyme in relation to pH and temperature. Meat Science, 69(1), 143-149.*

The planning of this study was carried out by M.Sc. Maria Ylä-Ajos (née Kylä-Puhju) and Prof. Eero Puolanne. The experimental study, including empirical work, data analysis and preparation of the manuscript was carried out by M.Sc. Ylä-Ajos. The study was supervised by Dr. Marita Ruusunen and Prof. Puolanne, both also participated in the preparation of the manuscript by giving comments and suggestions.

II *Ylä-Ajos, M., Ruusunen, M. & Puolanne, E. (2006). The significance of the activity of glycogen debranching enzyme in glycolysis in porcine and bovine muscles. Meat Science, 72(3), 532-538.*

The planning of this study was carried out by M.Sc. Maria Ylä-Ajos and Prof. Eero Puolanne. The experimental study, including empirical work, data analysis and preparation of the manuscript was carried out by M.Sc. Ylä-Ajos. The study was supervised by Dr. Marita Ruusunen and Prof. Puolanne, both participated in the preparation of the manuscript by giving comments and suggestions.

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The planning of this study was carried out by M.Sc. Maria Ylä-Ajos and Prof. Eero Puolanne. The experimental study, including empirical work, data analysis and preparation of the manuscript was carried out by M.Sc. Ylä-Ajos. The study was supervised by Dr. Marita Ruusunen and Prof. Puolanne, both of whom also participated in the preparation of the manuscript by giving comments and suggestions.

IV *Ylä-Ajos, M., Lindahl, G., Young, J.F., Theil, P.K., Puolanne, E., Enfält, A.-C., Andersen, H.J. & Oksbjerg, N. (2006). Post-mortem activity of the glycogen debranching enzyme and change in the glycogen pools in porcine M. longissimus dorsi from carriers and non-carriers of the RN⁻ gene. Meat Science. In press.*

The planning of this study was carried out by Dr. Gunilla Lindahl, M.Sc. Maria Ylä-Ajos and Research Prof. Niels Oksbjerg. The sampling was carried out by Dr. Lindahl and Dr. Ann-Charlotte Enfält and the design of the primers and probes used was done by Dr. Peter Theil. Most of the empirical work and preparation of the manuscript were carried out by M.Sc. Ylä-Ajos and the data analysis by M.Sc. Ylä-Ajos, Dr. Theil and Research Prof. Oksbjerg. The study was supervised by Prof. Eero Puolanne and Research Prof. Oksbjerg. Besides the supervisors Dr. Lindahl, Dr. Jette Young, Dr. Theil and Research director Henrik Andersen participated in preparation of the manuscript by giving comments and suggestions.

1 INTRODUCTION

Glycogen is the principal storage form of carbohydrate in animal species and it is more or less abundant in nearly all types of animal cells (Nelson & Cox, 2005). The two largest depot sites of glycogen storage are the skeletal muscles and the liver. The function of liver glycogen is to maintain the blood glucose levels within the required range (Roach, 2002), whereas muscle glycogen functions solely as a fuel reserve for the generation of ATP needed in muscle contraction (Nelson & Cox, 2005).

In living muscles, ATP is maintained at a constant level (homeostasis) and several energy sources can be called up during muscle contraction. The most economical pathways are the aerobic processes: oxidative phosphorylation via Krebs cycle. Aerobic energy production is accompanied by anaerobic processes: (a) the conversion of a high energy phosphate, phosphocreatine (PCr), and adenosine diphosphate (ADP) to creatine and adenosine triphosphate (ATP), (b) degradation of carbohydrates to lactate and (c) the myokinase reaction, by which 2 ADP's unite to form one ATP and one adenosine monophosphate (AMP) molecule. Glycogen is rapidly made available for both brief muscle activity and the early stages of sustained activity. It can be utilized aerobically and anaerobically. The relative intensity and duration of the physical stresses on different types of muscle fibres determine their respective patterns of glycogen depletion (Essén, 1978; Vøllestad & Blom, 1985; Asp et al., 1999; Essén-Gustavsson et al., 2005).

The breakdown of carbohydrates is the most important energy source for muscles *post mortem* (Bendall, 1973). The mechanism for carbohydrate catabolism is the same in living muscle and muscle *post mortem*. However, in the latter the reactions are irreversible and continue further (Lawrie, 1985). The oxygen content of muscles depletes within a few minutes *post mortem* and the ATP is fully replenished by the: breakdown of phosphocreatine and degradation of carbohydrates via anaerobic pathways (Bendall, 1973). The tendency of muscle fibres to maintain the ATP at constant levels after death of an animal leads to the process whereby muscle is converted into meat. The splitting of ATP to ADP plus P_i and proton (H^+) determines the rate and magnitude of carbohydrate catabolism. Without this reaction glycolysis and acidification of muscles would then come to a stop very rapidly (Bendall, 1973). Thus, the rate of pH decrease reflects the intensity of the muscle metabolism *post mortem*. Different species as well as different muscles within the same species have unique pH curves *post mortem*. The energy production *post mortem* results in an acidification of the muscles at pH values between 5.4 to 6.2 (Bate-Smith, 1938; Hamm, 1977; Bendall & Swatland, 1988; Offer & Knight, 1988b; Monin, 2004; Robergs et al., 2004) depending on the glycogen content, structural buffering capacity and the type of muscle.

Glycogen is spherical, highly branched polysaccharide, which comprises glucose chains that are organized into concentric tiers (Gunja-Smith et al., 1970; Goldsmith et al., 1982; Meléndez-Hevia et al., 1993; Roach, 2002). Glycogen particles include most of the enzymes needed for its metabolism *in vivo* (Madsen & Cori, 1958; Meyer et al., 1970; Nelson et al.,

1972; Polishchuk et al., 1995; Lees et al., 2004). When glycogen is degraded, the enzyme glycogen phosphorylase releases glucose as glucose-1-phosphate (G-1-P) from the non-reducing ends of the glucose chains in the outermost concentric tier of the glycogen particle extremely rapidly (Newsholme & Start, 1979). The action of glycogen phosphorylase stops when it reaches the fourth glucose unit from the branching point of the glucose chain (Walker & Whelan, 1960; Meléndez-Hevia et al., 1993). Glycogen debranching enzyme (GDE) breaks down the branching point making the glycogen molecule again susceptible to the action of glycogen phosphorylase (Brown & Illingworth-Brown, 1966; Hers et al., 1967; Taylor & Whelan, 1968; Nelson et al., 1969). Thus, the glycogen degradation is accomplished by the combined action of phosphorylase and glycogen debranching enzyme acting in concert.

Despite the extent of pH decrease and the ultimate pH value (pH_u) attained after slaughter, some glycogen always remains unconverted to lactate in muscles after the *post mortem* reaction sequence has ended ((Lawrie, 1955; Lawrie et al., 1959; van Laack & Kauffman, 1999; Immonen & Puolanne, 2000). It has been suggested that several factors including: adenosine monophosphate deficiency, a shortage of ADP or glucose and inhibition of glycolytic enzymes by low pH (Kastenschmidt et al., 1968; Scopes, 1971; Lundberg et al., 1987; Pearson & Young, 1989a; van Laack et al., 2001; Rhoades et al., 2005) may stop *post mortem* glycolysis. The glycolysis may also stop in some situations as a result of insufficient GDE action, leading to a decrease in glucose units easily accessed by phosphorylase. The metabolism of carbohydrate in meat production animals has been reviewed recently by Pösö and Puolanne (2005). The *post mortem* catabolism of carbohydrates is particularly interesting because both the rate and the extent of pH decrease have significant effects on both technological and sensory qualities of meat (Briskey, 1964; Hamm, 1974; Bendall & Swatland, 1988; Offer & Knight, 1988a,b; Pearson & Young, 1989a).

2 REVIEW OF LITERATURE

2.1 Carbohydrate catabolism

Adenosine triphosphate (ATP) is a short term energy supply of cells. It is the major carrier of chemical energy in all cells. In the muscle, ATP is maintained at constant level (homeostasis), and the muscle is also able to perform this task for a while after slaughter (Bendall, 1973).

2.1.1 Carbohydrate catabolism in muscles

The concentration of ATP in the muscle usually lies between 4 to10 mmol/kg muscle w.w., depending on the animal species and the muscle type (Tarrant et al., 1972; Bendall, 1973, Bendall, 1975; Richter et al., 1982; Renou et al., 1986; Bangsbo et al., 1992; Henckel et al., 2002). There are several ATP hydrolases (ATPases) present in skeletal muscles such as: myosin ATPase, sarcoplasmic reticulum Ca^{2+}-ATPase, mitochondrial ATPase and ATPases of the sarcoplasmic reticulum which are activated by Na^{+}, K^{+} or Ca^{2+} ions (Bendall, 1973; Pearson & Young, 1989a; Nelson & Cox, 2005). The energy released by the action of these enzymes is mainly used for muscle contraction, keeping the cytoplasmic Ca-content low and maintaining the balance between Na and K.

Breakdown of carbohydrates is one of the most important energy sources for ATP production in muscles. In living muscles, blood glucose and muscle glycogen provide the major part of glucosyl units (Hocquette et al., 1998). Carbohydrates are utilized aerobically and also anaerobically. Glycogenolysis is the breakdown of glycogen to G-1-P and glucose whereas glycolysis is the stepwise degradation of G-1-P and glucose, which eventually ends with pyruvate. Pyruvate is the junction point in carbohydrate metabolism. When oxygen is available and the density of mitochondria is not a limiting factor, pyruvate enters a mitochondrion and is converted to acetyl-CoA and further oxidised in the Krebs cycle. Under anaerobic conditions, Krebs cycle is halted and the lactate dehydrogenase catalyses the reduction of pyruvate to lactate in fibre cytosol (Newsholme & Leech, 1983).

Primarily anaerobic degradation of glycogen to lactate is a rapid metabolic source of ATP, which makes swift movements possible. Anaerobic energy production by glycolysis allows a fast adjustment to an increased energy demand, since it can be accelerated faster than ATP production from oxidative phosphorylation (Juel, 1997). Pösö and Puolanne (2005) calculated that the pig and the bovine both increased their respective muscle oxygen consumption during maximal exercise to near 30 ml/kg/min and 50 ml/kg/min. These oxygen consumption levels are 10- and 20-fold increases over their resting levels. However, the energy consumption may increase by as much as 100-fold over their resting level. After one minute of intensive exercise, ATP was used maximally at rates between 200 to 300 mmol/kg muscle/min (Lister, 1988). The skeletal muscle capacity for anaerobic energy transduction is fully capable of

meeting the increased need of ATP (Newsholme & Leech, 1983; Karlström, 1995; Pösö et al., 2004). It has, as estimated from the maximum activities of glycogen phosphorylase, phosphofructokinase and aldolase, the potential to increase the rate of anaerobic glycolysis to about 100 mmol lactate/kg/min (Scopes, 1970). In living heavily working muscle, despite the rapid anaerobic energy production part of the energy consumed is always produced aerobically.

Nevertheless, anaerobic energy production is an inefficient way to produce ATP compared with aerobic oxidation. The complete aerobic oxidation of glucose-6-phosphate (G-6-P) yields 37 molecules of ATP whereas only 3 molecules of ATP are regenerated anaerobically. Another disadvantage of anaerobic energy production is the excessive production of lactate and protons leading to a decrease in muscle pH and eventually to fatigue. Metabolic acidosis occurs when ATP demand (i.e. the rate of ATP hydrolysis) exceeds the rate at which ATP is produced in the mitochondria (Bendall, 1973; Honikel & Hamm, 1974; Hamm, 1977; Robergs et al., 2004). Thus the rate and quantity of muscle H^+ production (pH decrease) are consequences of ATP hydrolysis coupled with glycolysis. Lactate formation is a prerequisite for continuing anaerobic energy production. Lactate formation maintains the rate of anaerobic glycolysis by maintaining a redox equilibrium between NAD^+ and NADH (Nelson & Cox, 2005). Furthermore, lactate production actually retards muscle acidosis, which is a fact that goes against the generally held belief that lactate formation induces acidosis (Bendall, 1973; Honikel & Hamm, 1974; Robergs et al., 2004). Even if there is no causal relationship between lactate production and coincident pH decrease, lactate production is still a good indirect marker for fibre metabolic conditions that induce increased proton release and metabolic acidosis.

The initial anaerobic phase occurs at the onset of stimulation when peak tensions are generated and the ATP demand is higher than the aerobic capacity. According to Lister (1988) the ATP present in muscle would support intense contractions for about 1 to 5 seconds in the absence of ATP regeneration. Phosphocreatine (PCr) is rapidly degraded to regenerate ATP and the *in vivo* PCr content of between 20 to 25 mmol/kg (Tarrant et al., 1972; Bendall, 1973; Bendall, 1975; Henckel et al., 2002) is enough to support 4 to 5 seconds of intense activity (Lister, 1988). At a concentration of 80 mmol/kg of muscle, glycogen is the equivalent of approximately 240 µmol ATP/g produced anaerobically. Such a concentration is sufficient to support maximal muscle activity for 70 seconds (Lister, 1988). The standpoint above is very theoretical. However, quite short but intensive anaerobic activity prior to slaughter may significantly and rapidly reduce muscle glycogen content.

Small amounts of lactate (5 to 16 mmol/kg) are produced almost constantly in the muscles of living resting animals and resting man (Bendall, 1973; Richter et al., 1982; Fernandez et al., 1992a, 2002; Henckel et al., 2002). In living muscle lactate can be used for energy production, it is not a waste product *per se*. When oxygen is available, lactate is oxidized for energy in the heart, brain, liver, dark (oxidative) muscles, and to a lesser extent in light (glycolytic) muscles (Juel, 1997). Some lactate is also transported to liver and converted back to glucose. The conversion from lactate to pyruvate and *vice versa* is catalysed by lactate dehydrogenase (LDH), which in most tissues occurs in five different isoenzymes (Markert, 1963). LDH tetramers are composed of two types of subunits, M and H, which determine the

action of the enzyme. LDH-1 (H_4) is predominant in heart muscle and tends to favour rapid oxidation of lactate to pyruvate. On the other hand, the predominant type in skeletal muscle is LDH-5 (M_4), which favours the rapid reduction of low concentrations of pyruvate contents to lactate. It is also a key enzyme for the conversion of pyruvate to lactate *post mortem*. Porcine muscles have an exceptionally high LDH activity (Hamm & El-Badawi, 1991; Suuronen, 1995).

2.1.1.1 The determination of fuel utilization in muscle

The energy metabolism in skeletal muscles is regulated in several ways. Hocquette et al. (1998) reviewed the nutritional and hormonal regulation of energy metabolism in meat production animals. The physiological state of a muscle influences which chemical form the fuel is mainly used (Hultman, 1995). A resting or only slightly working muscle satisfies its need for energy by oxidising fatty acids and blood glucose and the actual fuel source used is determined by substrate availability. In exercising muscle, the relative intensity and duration of the physical activity determine the source of energy (Hultman, 1995). When the relative intensity of the physical activity is increased, the aerobic oxidation of glucose or fatty acids becomes insufficient to meet the need of ATP re-synthesis, and glycogen may be oxidized to provide additional energy (Newsholme & Leech, 1983; Hultman, 1995). When muscle energy demand further increases and exceeds the aerobic capacity of the muscle, glycogen is degraded anaerobically and extra ATP is obtained from glycolysis (Newsholme & Start, 1979; Newsholme & Leech, 1983). Glycogen and phosphocreatine (PCr) are the dominant fuels for ATP production during short-term intense exercise (Roach, 2002).

The net rate of glycogenolysis increases with exercise intensity but often decreases with exercise duration (Bergström et al., 1971; Essén, 1978; Graham et al., 2001). Fridén et al. (1989) concluded that, there are differences in the sequential glycogen utilization patterns depending on the type of exercise. During short term intense exercise, glycogen availability has no effect on the rate of glycogenolysis or the ability to generate and maintain maximal power output in rat or human muscle (Sahlin et al., 1989; Ren et al., 1990; Spriet et al., 1990; Bangsbo et al., 1992; Hargreaves et al., 1997). Nevertheless, the ability to maintain submaximal exercise and endurance exercise are positively correlated with the pre-exercise glycogen content (Bergström et al., 1967; Galbo et al., 1979; Richter & Galbo, 1986; Hespel & Richter, 1990, 1992; Vandenberghe et al., 1999; Shearer et al., 2001). Consistent with this, Essén-Gustavsson et al. (2005) found that pigs with the RN^- mutation (high glycogen content) were able to sustain submaximal exercise for longer periods than the wild type pigs (normal glycogen content) whereas there was no significant difference in the rate of glycogenolysis between the genotypes. Hespel and Richter (1990) showed that when glycogen content is low, muscles increase their glucose uptake. Even so, it is still not clear if the low glycogen levels *per se* or some other factors are responsible for the decline in the glycogenolytic rate. It has been shown that preceding exercise, rather than low glycogen content, causes a significant reduction in performance when the exercise is performed a second time (Grisdale et al., 1990; Bangsbo et al., 1992). In man, prolonged vigorous exertion can cause almost total depletion of glycogen (Bergström et al., 1967).

2.1.1.2 Muscle pH

The pH of a living muscle at rest is between 7.0 and 7.3 (Tarrant et al., 1972; Bendall, 1973; Bendall, 1975; Renou et al., 1986; Kivikari, 1996; Kylä-Puhju et al., 2004). However, the pH value of muscle with zero lactate seems to be higher in fast twitch glycolytic (FG) muscles than in slow twitch oxidative (SO) muscles (Kylä-Puhju et al., 2004). Muscle pH is kept at a level that allows an effective function of vital enzymes. Homeostasis is maintained by strong metabolic and structural buffering (Bate-Smith, 1938; Davey, 1960; Bendall, 1973; Rao & Gault, 1989; Kivikari, 1996). It is also maintained by transporting protons together with lactate out of the fibre mainly with help of monocarboxylate transporters and Na^+/H^+ exchange (Juel, 1996; Juel, 1997; Halestrap & Price, 1999; Sepponen et al., 2003). Thus, several buffer systems prevent a rapid fall of pH in exercising muscle where energy is produced by anaerobic glycolysis. Nevertheless, the pH of a fatigued muscle may fall to as low as 6.5 or even 6.3 (Lovell et al., 1987; Juel, 1996). Moreover, stress immediately pre-slaughter reduces the pH of the muscle while the animal is still alive (Tarrant et al., 1972; Henckel et al., 2000) and the pH of muscle at the time of slaughter is usually lower than at rest.

2.1.2 Carbohydrate catabolism in meat

There are two enormous differences between the catabolism of muscle carbohydrates *pre mortem* and *post mortem*: (1) the oxygen supply to the fibres ceases soon after the blood circulation has been interrupted by the death of the animal and (2) the end-products of glycolysis are not carried out from the muscle for the same reason. The mechanism for carbohydrate catabolism is the same in living and in *post mortem* muscle. However, in the latter the reactions are irreversible and continue further (Lawrie, 1985).

The main bulk of muscles become anaerobic within a few minutes after death (Bendall, 1973). Pösö and Puolanne (2005) calculated that the oxygen stored in myoglobin allows the aerobic production of 0.4 to 6 mmol ATP, depending on animal species, age and muscle type. After the oxygen has been used, ATP is replenished by fully anaerobic pathways in which the breakdown of PCr and the degradation of glycogen occur. Maximally, 20% of the ATP consumed *post mortem* is produced from PCr (Pösö & Puolanne, 2005) and the rest is derived from anaerobic glycolysis.

The regulatory enzymes that control ATP metabolism and glycolysis in the living tissue are still active in the muscle *post mortem*, but are able to maintain the *ante mortem* levels of ATP for only as long as the PCr supply lasts (Bendall, 1951; Lawrie, 1953; Tarrant et al., 1972; Bendall, 1973; Bertram et al., 2002). When the level of PCr is reduced to approximately 25% of the resting value, a decrease in glycogen, in ATP content and in pH will be observed with simultaneous increases in lactate, IMP, inosine, hypoxanthine and P_i formation, while the content of sugar phosphates remains relatively constant (Bendall, 1951; Lawrie, 1953; Bendall, 1973; Asghar & Pearson, 1980; Lundberg et al., 1987; Schäfer et al., 2002; Christensen et al., 2004). Within a few hours *post mortem* the ATP levels will have

decreased to about one third of the resting value (Bendall, 1951; Bendall, 1973), and the onset of *rigor mortis* ensues (Bendall, 1951; Lawrie, 1953; Bendall, 1973).

2.1.3 The rate of *post mortem* carbohydrate catabolism

Anaerobic energy production *post mortem* results in an acidification of the muscles. The rate of muscle pH fall depends on the rate of H^+ formation due to ATP hydrolysis coupled with glycolysis (Scopes, 1971; Bendall, 1973; Honikel & Hamm, 1974; Hamm, 1977; Robergs et al., 2004). The characteristics of the pH decease are determined by the physiological condition of the muscles at the time of stunning. A relatively slow rate of glycolysis and a moderately low pH_u (about 5.4) are characteristic of normal muscle, usually resulting in tender meat (Asghar & Pearson, 1980).

The rate of pH decrease reflects the intensity of the *post mortem* muscle metabolism. It is influenced by: (1) variation in stress that animals undergo before and during the slaughter process (2) variations in metabolic and contractile mechanisms, including species, breed and muscle differences, availability of carbohydrates and oxygen (3) variation in the location of the muscles on the carcass leading to variations in temperature changes (Bendall, 1973; Asghar & Pearson, 1980; Pearson & Young, 1989a; Monin & Ouali, 1991). Thus, different muscles from the same species have different pH curves *post mortem*. For example, the rate of pH decrease is higher in the *M. semimembranosus* than it is in the *M. longissimus dorsi* or *M. psoas major* in the cattle and the pig (Pearson & Young, 1989a; Henckel et al., 2000). The rate of *post mortem* glycolysis is faster (Beecher et al., 1965a; Beecher et al., 1965b; Lefaucheur et al., 1991) and the pH_u value is lower in the FG muscles of the pig (Beecher et al., 1965b; Laborde et al., 1985; Monin et al., 1987; Bendall & Swatland, 1988; Warner et al., 1993; Przybylski et al., 1994; Enfält et al., 1997b), the cattle (Hunt & Hedrick, 1977; Talmant et al., 1986; Rao & Gault, 1989; Przybylski et al., 1994) and of poultry (Kijowski et al., 1982; Jones & Grey, 1989) than in the respective SO muscles of these species.

Slaughtering causes a marked drop in the pH and the contents of PCr and glycogen of unanesthetized muscle (Bendall, 1973; Pearson & Young, 1989a; Bertram et al., 2002; Henckel et al., 2002), since stunning will cause an excitation phase involving involuntary contractions of the muscles in the animals (Bendall, 1973). Captive bolt stunning is the most violent stunning method and therefore most likely to cause accelerated pH decrease (Bertram et al., 2002). Electrical stunning accelerates *post mortem* muscle metabolism more than gas stunning in poultry and pigs (deFremery & Pool, 1960; Barton-Gade, 1997; Henckel et al., 1998; Bertram et al., 2002). However, electrical stunning slowed down *post mortem* metabolism in chickens compared to those chickens that were not stunned at all (Lee et al., 1979).

Generally the rate of *post mortem* pH decrease follows the order poultry>pork>lamb>beef in the main meat species (Lundberg et al., 1987; Pearson & Young, 1989a; Warriss, 2000; Monin, 2004). Furthermore, ATPase activity is higher in fast glycolytic muscles than in slow oxidative muscles (Laborde et al., 1985). In poultry, the rate of *post mortem* pH fall is very

fast, 0.03 units/min has been reported for turkey breast muscle (Pearson & Young, 1989a; Sosnicki et al., 1998) and 0.0067 units/min for chicken (Young et al., 2002). The rate of decrease of pH is very variable between pig breeds and between individual pigs (Briskey & Wismer-Pedersen, 1961; Kastenschmidt et al., 1968; Sellier & Monin, 1994; Henckel et al., 2000). In the non-stimulated porcine *longissimus dorsi* muscle the rate of pH decrease may vary from 0.003 to 0.017 pH units/min (measured less than 120 min post slaughter) (Bendall et al., 1963; Tarrant et al., 1972; Bendall & Swatland, 1988; Miri et al., 1992; Henckel et al., 2000). The fastest rates of pH decline early *post mortem* are found in PSE prone muscles producing PSE meat (Bendall & Swatland, 1988). In a review by Sosnicki et al. (1998) extreme values of 0.06 pH units/min for PSE turkey and even 0.1 pH units/min for PSE pig muscle were reported. The rate of decrease in pH is generally low in cattle and sheep ranging from 0.0045 to 0.0067 units/min at 37 °C (Pearson & Young, 1989a). Hence, the pH_u values in extreme cases are sometimes reached in as little as one hour, whereas usually this process takes between 6 and 12 h in pigs and 18 to 40 h in cattle (Honikel, 1992).

A fast rate of carbohydrate catabolism just before stunning and early *post mortem* increases muscle temperature (Bowker et al., 1999) leading to a faster rate of pH decrease. Reduced pre-slaughter stress results in lower carcass temperature post-slaughter, which again has a positive effect on meat quality (Tarrant & Lacourt, 1984; Støier et al., 2001), and may decrease the development of PSE (pale soft exudative) meat. The rate of glycolysis decreases with decreasing temperature (from 43 ºC to 5 ºC) (Bendall, 1951; Bendall, 1973; Jolley et al., 1981) and with increasing time *post mortem* (Bendall, 1973; Jolley et al., 1981; Schäfer et al., 2002; Young et al., 2002). Thus, *post mortem* glycolysis will tend to be fast in muscles that are slow to cool. However, the rate of glycolysis rises again in temperatures near to zero degrees, due to an inhibition on the action of calcium pumps and following increases in the Ca^{2+} content of the muscle fibres (Bendall, 1973; Jolley et al., 1981). Moreover, low temperatures have an especially strong influence on slow twitch oxidative muscles.

It has been shown that the animal's genotype, the type and the amount of stress imposed on the animals during pre-slaughter handling are major causes of the variations observed in pH decline (Warriss, 1987; Fernandez & Tornberg, 1991; Sellier & Monin, 1994; Warner et al., 2001). In particular, the presence of the halothane gene (Hal) and the PRKAG3 gene (RN-gene, acid meat) in pig (Sellier & Monin, 1994; Rosenvold & Andersen, 2003; Monin, 2004) are of major importance, since both have an influence on energy metabolism in living muscles and also in muscles *post mortem* (Monin et al., 1986).

The pH decrease *post mortem* is faster in porcine and chicken muscles containing high levels of glycogen compared to when the muscles of these species contain low levels of glycogen (Warriss et al., 1988; Henckel et al., 2000; Henckel et al., 2002; Berri et al., 2004). Also the structure of glycogen is important factor for *post mortem* pH formation. In early studies, the highest rates of glycolysis were found in muscles where the external chains of glycogen were the longest (Lawrie et al., 1959; Sayre et al., 1963b). High muscle PCr content at the moment of slaughtering implies slower decreases in pH (Bendall, 1951; Lawrie, 1953; Bendall, 1973; Hamm, 1977; Bertram et al., 2002; Henckel et al., 2002; Pösö & Puolanne, 2005), since in the breakdown of PCr, one proton is bound to every creatine molecule produced (Nelson & Cox, 2005). The phosphofructokinase enzyme is considered to be the

rate-limiting factor in glycolysis (Nelson & Cox, 2005). However, it has also been suggested that the rate of pH decrease is determined by the specific ATPase activity (Bendall, 1973; Scopes, 1974; Hamm, 1977) rather than the amounts of glycolytic enzymes (Scopes, 1974; Allison et al., 2003). Honikel and Hamm (1974) stated that the drop in pH at about pH 7.0, results from H^+ being liberated during the hydrolysis of ATP to ADP, whereas at pH 5.5 to 6.0, H^+ ions are released as a result of glycolysis.

2.2 Glycolytic and oxidative muscles

Almost all mammalian skeletal muscles are composed of three different fibre types, which have different characteristics such as: size, metabolic and contractile properties, capillary supply and many other features, depending on the function of the muscle (Monin & Ouali, 1991). The ratio of different fibre types varies between animals and also between muscles (Monin et al., 1987; Oksbjerg et al., 1994; Ruusunen & Puolanne, 2004). Generally, the muscles of the hindquarters are more contractile and have higher glycolytic activities than the muscles of the forequarters and probably have a more dynamic role to play than the latter (Talmant et al., 1986). Muscles that are involved in maintaining posture, or carry out slow repetitive movements or are used frequently, have a higher proportion of type I fibres than muscles that are more seldom in use (Goldspink, 1983).

Pearson and Young (1989b) have reviewed different methods for classifying muscle fibres and muscles. One approach is to classify muscles into three groups according to the contractile and metabolic characteristics of their main fibre type: fast twitch and glycolytic (FG), slow twitch and oxidative (SO), fast twitch and oxidative (FOG). This categorization corresponds closely to classifying muscle fibres according to IIB, I and IIA types. In living muscles, SO fibres tend to maintain their energy-rich phosphate levels aerobically, whereas FG and FOG muscles have a greater capacity for maintaining their ATP supply anaerobically (Pearson & Young, 1989b). The influence of fibre type distribution on meat quality has been reviewed by Karlsson et al. (1999) and Klont et al. (2001).

Different muscles are adapted to different types of exercise. On one hand, SO muscles are slow to respond to contraction stimuli, are efficient, economical, hydrolyze ATP only slowly, and are very fatigue resistant (Goldspink, 1983). On the other hand, a typical FG muscle responds rapidly to a single stimulus because of its rich and well developed sarcoplasmic reticulum. Moreover, FG muscles have high myosin ATPase specific activities and they contract intensively for short periods, but fatigue easily owing to their inability to replenish energy stores while contracting (Goldspink, 1983; Talmant et al., 1986; Pearson & Young, 1989b). In particular, FG muscles are used when rapid movement is required and their metabolism is well adapted to such a role. Consequently, glycogen degradation can occur extremely rapidly in FG muscles (Newsholme & Start, 1979). Similarly, FOG muscles are adapted for fast movements of a repetitive nature and their metabolism is intermediate between FG and SO muscles (Pearson & Young, 1989b).

The capacity of aerobic metabolism is limited by oxygen delivery and by the capacity of muscle fibres to use oxygen. For example, SO muscles have more blood capillaries, smaller diameters and higher contents of both myoglobin and mitochondria (Beecher et al., 1965b; Essén, 1978; Goldspink, 1983; Saltin & Gollnick, 1983; Laborde et al., 1985; Valberg & Essén-Gustavsson, 1987; Pearson & Young, 1989b; Ruusunen, 1994; Ruusunen & Puolanne, 2004) than FG muscles. Most molecular oxygen is bound to myoglobin in muscles. The content of myoglobin is strongly age-dependent in that it increases from early life to maturity (Pearson & Young, 1989b; Oksbjerg et al., 2000). The myoglobin content is generally higher in bovine than in porcine muscles (Beecher et al., 1965b; Pearson & Young, 1989b; Hamm & El-Badawi, 1991). In meat animals the aerobic capacity decreases in the following order: cattle > pig > poultry (Hocquette et al., 1998; Pösö & Puolanne, 2005).

Thus, the SO muscles have a high capacity for aerobic oxidation of glucose (Beecher et al., 1965b; Laborde et al., 1985; Talmant et al., 1986; Pearson & Young, 1989b) or fatty acids and they contain more fat (Beecher et al., 1965b; Hunt & Hedrick, 1977) than the FG muscles. In comparison, FG muscles are predominantly glycolytic i.e. they usually contain much glycogen, a high potential for amplification of the glycogenolytic cascade and high activity of phosphorylase, GDE and glycolytic enzymes (Briskey & Wismer-Pedersen, 1961; Dalrymple et al., 1973; Cohen, 1978; Newsholme & Start, 1979; Saltin & Gollnick, 1983; Tsutou et al., 1985; Monin et al., 1987; Lundström et al., 1989; Pearson & Young, 1989b; Petersen et al., 1997; van Laack et al., 2001), which allow a rapid conversion of glycogen into lactate (Valberg & Essén-Gustavsson, 1987).

In general, FG muscles are large, have large-diameter fibres and sparse capillarization, and can perform either intense exercise of short duration or prolonged, low level anaerobic functions (Castellini & Somero, 1981; Pösö & Puolanne, 2005). Protons are easily accumulated in FG muscles due to their anatomy and physiology. However, the capacity of FG muscles to maintain constant pH or resist a change in pH is higher than for SO muscles. The buffering capacity and the lactate-proton transport capacity in FG muscles are greater than in SO muscles (Juel, 1996; Kivikari, 1996; Juel, 1997; Sepponen et al., 2003). In living animal, during intense muscle activity and in the recovery period, the lactate-proton transport mechanism mediates the lactate and H^+ effluxes from the muscle fibres and thus reduces the drop in intracellular pH.

The genetic selection of meat production animals has mainly focused on productive traits such as, growth rate, feed conversion rate and lean meat percentage. It has been particularly successful in poultry and pigs because of these species' rapid reproduction rates. Unfortunately, genetic correlations between growth rate and meat quality traits; as well as between food conversion ratio and meat quality are generally unfavourable (Sellier & Monin, 1994). Brocks et al. (2000) showed that selection for either low back fat thickness or fast growth rate influenced on different properties of muscle fibre.

Presumably selection for leaner pigs and for a higher proportion of large muscle fibres causes changes both in fibre type distribution and in fibre size resulting in poor capillarization. Consequently, an insufficient delivery of oxygen and substrates and an insufficient elimination of end products, such as CO_2 and lactate, lead to a reduction in pork

quality (Essén-Gustavsson, 1992; Solomon et al., 1998; Karlsson et al., 1999). However, the results of different studies concerning the effects of breeding are not incontestable.

Selection for productivity traits and for rapid growth of lean muscle tissue leads to the selection of animals with a larger percentage of type IIB muscle fibres and increased fibre number. However, both an increase and also a decrease in circumference or cross-sectional area of the fibres have been reported (Rahelic & Puac, 1981; Solomon et al., 1998; Dransfield & Sosnicki, 1999; Brocks et al., 2000; Oksbjerg et al., 2000). The growth rate of type IIB fibres is two-fold higher than that of type I and type IIA fibres (Oksbjerg et al., 1994), but type IIB fibres have a low capacity for oxidative metabolism and therefore seem to release energy mainly through glycogenolysis (Essén-Gustavsson et al., 1988). With changes towards more glycolytic fibres, and an increased sensitivity to stress, a decrease in most meat quality characteristics might be expected (Henckel, 2002).

Prolonged selection (over a period of 19 years) of Danish Landrace pigs for high growth potential has led to an increase in the number of muscle fibres, an increase in the proportion of type IIB fibres and an increase in water content (Oksbjerg et al., 2000). However, selection also led to lower pigment levels, lower protein content and a lower proportion of type I fibres (Oksbjerg et al., 2000). Lonergan et al. (2001) reported that the selection for lean growth efficiency in pigs led to faster pH decreases early *post mortem* and an increase in the incidence of soft and exudative meat over those found in controls. Karlsson et al. (1993) observed no changes in fibre type composition in pigs fed a high protein diet and selected for lean tissue growth over four generations. In addition to a higher glycolytic capacity, these authors also reported that the selected pigs had a higher glycolytic and higher oxidative muscle metabolism than controls. On the other hand, Oksbjerg et al. (2000) reported a decrease in muscle glycolytic and oxidative enzyme activities, but stable glycogen levels, in Danish Landrace pigs during a prolonged breeding period in which growth potential was the selection criterion.

It seems that in poultry the limits of muscle growth rate have been reached (Solomon et al., 1998); the animals have been selected to a point where proper environmental conditions, optimal feeding strategies and handling of the animals are crucial to a positive economic production outcome. Chicken muscles are characterised by a very high number of glycolytic fibres (Papinaho et al., 1996; Mammoli et al., 2004) and poultry displays a higher sensitivity to stress in comparison to other meat producing animals (Young et al., 2002). It seems that the chickens are already metabolically exhausted at the time of sticking, resulting in high pH_u values. Young et al. (2002) reported that the breast muscle glycogen content at one minute after slaughter was reduced by 60% and lactate content increased by 330% compared to resting values. The incidence of PSE and DFD meat in poultry has increased (Barbut, 1997a, b; Sams & Alvarado, 2004). Lonergan et al. (2003) pointed out that besides growth rate there are marked differences in meat composition and quality characteristics between contemporary stocks (egg-type chickens and meat-type chickens). The present study, however, concentrates on meat-type chickens.

2.3 Muscle glycogen

2.3.1 The structure and location of muscle glycogen

The structure of glycogen is optimized to maximize the total glucose stored in the smallest possible volume. The structure of glycogen also maximizes the speed of fuel release by maximizing the proportion of glucose units that can be directly released by phosphorylase before any debranching occurs and the number of non-reducing ends (points of attack for phosphorylase) (Meléndez-Hevia et al., 1993). The glycogen formation compared to that if the glucose units were free in the cell, has a considerable effect of on osmotic pressure. Nelson and Cox (2005) stated that in liver the total glucose stored as glycogen is equivalent to 400 mM, whereas the content of glycogen is only 0.01 μM. However, liver glycogen is mainly stored in particles with a molecular weight of 10^7 Da, which is equivalent to about 55 000 glucose units in a molecule (Goldsmith et al., 1982). Thus, calculated from these values liver glycogen content is preferably 0.01 mM (K.-O. Honikel, personal communication). In muscles the glycogen content is one fifth or one tenth of that in liver, but even then glycogen formation has a great relevance on muscle functions.

Glycogen is a highly branched polysaccharide made up of D-glucose residues linked by α-1,4-glycosidic linkages between glucose residues to form long chains, which are branched by the formation of α-1,6-glycosidic linkages (Roach, 2002). Approximately 8% are α-1,6-links and the rest are α-1,4-links (Meléndez-Hevia et al., 1993). A self-glucosylating protein, glycogenin (E.C. 2.4.1.186, M.W. 37,300 Da) is located in the core of the glycogen molecule (Gunja-Smith et al., 1970; Goldsmith et al., 1982; Lomako et al., 1988; Alonso et al., 1995; Lomako et al., 2004). Branched glucose chains are attached to glycogenin. The structure and function of glycogenin is described in detail in a recent review by Lomako et al. (2004). The amount of glycogenin influences how much glycogen the cell can store (Alonso et al., 1995; Shearer et al., 2000). Starting from a glycogenin primer, glycogen is synthesised by two enzymes, glycogen synthase (EC 2.4.1.11) and glycogen branching enzyme (EC 2.4.1.18). The synthesis of glycogen has been reviewed in detail by Roach (2002).

The Whelan's model for glycogen (Gunja-Smith et al., 1970; Meléndez-Hevia et al., 1993) is generally accepted (Goldsmith et al., 1982). A diagram of glycogen representing the structure of a glycogen molecule is shown (Figure 1). Meléndez-Hevia et al. (1993) described the structure of a glycogen molecule. Glycogen is formed by two different kinds of glucose chains (chain length 12 to 14 glucose residues): branched B-chains and non-branched A-chains. Every B-chain has two branches on it, creating further A or B-chains. There are four glucose residues between branches and a tail of four glucose units after the second branch in the B-chains. In three dimensions the molecule is spherical and organized into concentric shells or tiers. Every B-chain lies in the inner tiers and all of the A-chains lie on the outermost tier. In the degradation of glycogen, glucose can be released from the non-reducing ends of all the A-chains at one time. Because the degree of branching is two, each subsequent tier going away from the centre of the molecule has twice the number of chains as the previous one, and as much glucose units than all the previous tiers together. The

advantage of such a high degree of branching is to provide large numbers of non-reducing ends susceptible for enzyme degradation. The fine structure of glycogen is described in more detailed in the following studies Goldsmith et al. (1982), Meléndez-Hevia et al. (1993), Meléndez et al. (1997).

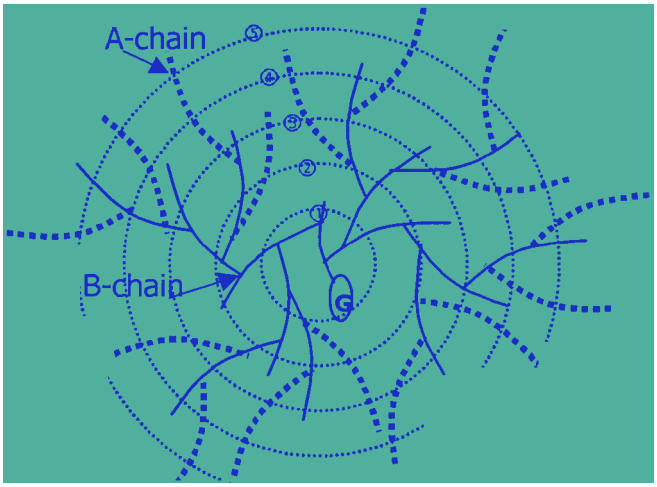

Figure 1. *The schematic structure of a glycogen particle (adapted from Immonen, 2000). The structure represents the Whelan's model of glycogen (Gunja-Smith et al., 1970); see also Goldsmith et al. (1982) and Meléndez-Hevia et al. (1993). Glycogenin (G) is found in the middle of the figure, the bold dotted lines represent A-chains and the solid lines B-chains of glycogen.*

In mammalian muscles, glycogen is organized into a structure called glycogen β-particles (Roach, 2002), with a particle diameter of between 10 to 40 nm (Estrade et al., 1993; Marchand et al., 2002). Glycogen particles include most of the enzymes needed for its metabolism *in vivo*, such as glycogen synthase, phosphorylase kinase, phosphorylase and glycogen debranching enzyme (Madsen & Cori, 1958; Meyer et al., 1970; Nelson et al., 1972; Taylor et al., 1975; Becker et al., 1977; Scraba et al., 1988; Polishchuk et al., 1995; Lees et al., 2004). A "mature" glycogen β-particle (macro-glycogen particle) is a spherical particle arranged in 12 concentric layers (tiers) of carbohydrate, has a maximal diameter of 42 nm and a molecular mass of 10^7 Da, equivalent to about 55000 glucose residues (Goldsmith et al., 1982; Meléndez-Hevia et al., 1993; Meléndez et al., 1997). Macro-glycogen and pro-glycogen are explained in detail in the next section. The size of the glycogen molecule is self-limiting for two reasons. First, the density of glucose residues in the outermost tier prevents further growth and second, enzymes within the molecule occupy space (Madsen & Cori, 1958; Goldsmith et al., 1982; Meléndez-Hevia et al., 1993).

Glycogen differs from most of the other important compounds of the body in not having a fixed molecular weight. It is continuously being synthesized and degraded at rest and during exercise in a substrate cycle (Bonen et al., 1985; Hutber & Bonen, 1989; Azevedo et al., 1998). Thus, the concept of glycogen content is in itself a difficult one to determine because each glycogen molecule changes the effective concentration of glucose at its outer branches as its size is reduced. Usually glycogen content is expressed as the content of glucose units that are bound in it.

Fridén et al. (1989) found that muscle glycogen is stored at five topographically different locations: at the subsarcolemmal space (in proximity to mitochondria), the intermyofibrillar space (adjacent to the mitochondria), the para-Z-disc, the N_2-line (the lateral end of the I-band) and the H-zone spaces. Furthermore, Estrade et al. (1993) and Marchand et al. (2002) found that the largest glycogen compartment is the intermyofibrillar space, though the subsarcolemmal space is more densely packed with glycogen granules.

2.3.2 Pro-glycogen and macro-glycogen

Biochemical studies (Lomako et al., 1991; Lomako et al., 1993) have shown that glycogen exists in two forms in skeletal muscle: acid-soluble, high molecular weight macro-glycogen (MG) (which has a M.W. 10^7 Da) and acid-insoluble, low molecular weight pro-glycogen (PG) (which has a M.W. 400,000 Da). The early reports on both glycogen forms have been reviewed by Stetten and Stetten (1960). The greater solubility of MG over that of PG is a result of its lower protein to carbohydrate ratio, 0.35% protein and 10% protein, respectively (Lomako et al., 1991). It has been estimated that the MG molecule contains approximately 25 times more glucosyl units per glycogenin molecule than the PG molecule (Alonso et al., 1995). The number of non-reducing ends can be calculated on the basis of molecular weight. Consequently, MG has about 2100 non-reducing ends from which the release of glucose may begin simultaneously, whereas in the PG form there are only between 64 to 128 non-reducing ends per molecule (Pösö & Puolanne, 2005).

When studying the distribution of glycogen molecules by electron microscope Fridén et al. (1989) reported the existence of two glycogen populations with distinctly different diameters. In contrast, Marchand et al. (2002) found that muscle glycogen diameters follow a continuous normal distribution, which implies that pro-glycogen is not a distinct entity, but rather that PG and MG fall into various categories on the basis of the sizes of their molecules. Both glycogen pools (MG and PG) are suitable substrates for energy production during contraction of skeletal muscle (Huang et al., 1997; Derave et al., 2000), but both pools seem to be metabolized separately (Graham et al., 2001; Shearer et al., 2001). It has even been suggested that the enzymes (glycogen synthase, phosphorylase, branching and debranching enzymes) of both glycogen pools might be distinct from each other (Lomako et al., 1993; Alonso et al., 1995; Meléndez et al., 1997). The subcellular location and the density of branches in the outer tiers of the molecules may be important factors as to which order the glycogen pools are utilized (Shearer et al., 2001).

The PG pool accounts for approximately 65 to 75% of the total glycogen in resting human muscle with full glycogen stores (Adamo & Graham, 1998; Adamo et al., 1998; Asp et al., 1999; Graham et al., 2001; Shearer et al., 2001), however, the MG fraction increases with increasing muscle glycogen content. In pigs, the PG:MG ratios are quite similar than in human, PG represents approximately 50 to 72% and MG 28 to 50% of the total glycogen content, the variation being a function of the level of stress on the animal and its diet (Rosenvold et al., 2003; Essén-Gustavsson et al., 2005).

Which glycogen pool is utilized (PG versus MG) depends on the availability of glycogen and also the type of exercise. However, the results of different studies are quite conflicting and it seems that there might also be differences in glycogen utilization patterns between species. For example, in the horse during intense exercise both PG and MG seem to contribute equally to glycogenolysis (Bröjer et al., 2002). Derave et al. (2000) found that in rat muscles with normal to low initial glycogen contents, the content of PG is higher and it contributes more in absolute numbers to glycogenolysis than MG. However, MG seems to be more easily broken down, when freely available (Derave et al., 2000). On the other hand, Graham et al. (2001) and Shearer et al. (2001) reported that in human muscle with normal to high initial glycogen contents, PG is used in anaerobic very high-intensity exercise. In contrast, MG seems to be more resistant to mobilization than PG though it is also utilized during intense exercise (Graham et al., 2001). However, the degradation of PG rapidly ceases as the period of exercise is extended or the exercise is subsequently repeated (Graham et al., 2001). The degradation of MG predominates in aerobic long-lasting exercise in humans (Asp et al., 1999) and in pigs (Essén-Gustavsson et al., 2005). Rosenvold et al. (2003) reported that mainly PG degradation occurs in pig *longissimus dorsi* muscle during the first 45 min after slaughter.

Barber et al. (1967) showed that phosphorylase has a higher affinity for low molecular weight glycogen in the liver. These authors reported that approximately three times the amount of phosphorylase (on activity per gram basis) was associated with low molecular weight glycogen than with the high molecular weight form. However, the hepatic PG content is very low, approximately 3% of total glycogen in the liver (Alonso et al., 1995).

2.3.3 Glycogen content in different animals

The amount of glycogen in muscles varies between species and is higher in biopsy samples taken from living animals than those samples taken *post mortem* (Fernandez et al., 1992b; Enfält et al., 1997b; Le Roy et al., 2000; Miller et al., 2000; Fernandez et al., 2002; Henckel et al., 2002; Young et al., 2002). The glycogen content of chicken breast muscle is around 50 mmol/kg muscle (Lee et al., 1976; van Laack et al., 2000; Berri et al., 2004), but low values of 19.5 mmol/kg, shortly *post mortem* have also been reported (Young et al., 2002). Shortly after slaughter, the glycogen content in the FG *longissimus dorsi* muscle ranges from 60 to 100 mmol/kg w.w. in cattle (Crouse & Smith, 1986; Przybylski et al., 1994; Immonen et al., 2000c), and is around 80 mmol/kg in most pig breeds (Fernandez & Gueblez, 1992; Enfält et al., 1997b; Rosenvold et al., 2001; Fernandez et al., 2002).

Generally, the differences in glycogen content between pig breeds are small (Tarrant et al., 1972; Monin & Sellier, 1985; Monin et al., 1987; Suuronen, 1995; Henckel et al., 1997), with the exception of the Hampshire breed. The FG muscles of Hampshire pigs frequently contain markedly more glycogen than the muscles of other breeds (Sayre et al., 1963a; Sayre et al., 1963b; Dalrymple et al., 1973; Monin & Sellier, 1985; Monin et al., 1987; Fernandez & Tornberg, 1991; Suuronen, 1995; Enfält et al., 1997b). A dominant gene (PRKAG3 or RN$^-$

gene) was found in Hampshire pigs, which adversely affects meat quality (Fernandez et al., 1992b; Estrade et al., 1993; Sellier & Monin, 1994; Enfält et al., 1997b; Josell et al., 2003a, b; Lindahl et al., 2004). The RN^- carrier pigs have about 70% higher glycogen concentration in FG muscles than rn^+rn^+ homozygous pigs (Estrade et al., 1993; Enfält et al., 1997a; Le Roy et al., 2000; Essén-Gustavsson et al., 2005).

Sex does not have an influence on muscle glycogen content in the pig (Monin & Sellier, 1985; Fernandez & Tornberg, 1991; Suuronen, 1995) or in cattle (Immonen et al., 2000b). However, it appears that male animals are more prone to pre-slaughter glycogen depletion than castrates or females because of their excitable temperament and aggressive behaviour (Warriss, 1987; Warriss, 1990; Fernandez & Tornberg, 1991; Grandin, 1993; Monin, 2004).

2.3.4 Glycogen content in different muscles

The glycogen content varies between different muscles within the same animal. The GP is evidently related to the metabolic and contractile properties of the muscles. The highest levels of glycogen have been observed in the FG muscles of the pig (Dalrymple et al., 1973; Monin et al., 1987; Lefaucheur et al., 1991; Karlsson et al., 1993; Przybylski et al., 1994; Wittmann et al., 1994; Fischer & Dobrowolski, 2002), cattle (Talmant et al., 1986) and chicken (Warriss et al., 1988; Sams & Janky, 1991). The range of GP is great in different muscles of the pig, varying from 63 in SO *M. vastus intermedius* to 187 mmol/kg in FG *M. longissimus dorsi* (Fischer & Dobrowolski, 2002).

On the other hand, some studies have obtained the opposite results suggesting that the metabolic characteristics of a pig muscle do not influence its glycogen content or pH_u value (Beecher et al., 1965b; Essén et al., 1980; Fernandez et al., 1995). Different muscles respond in various ways to stress. Furthermore, the type of stress before slaughter has an influence on the extent of recruitment of different types of fibres (I, IIA and IIB), giving rise to different glycogen depletion patterns (Lacourt & Tarrant, 1985; Essén-Gustavsson, 1992). Stress (including fasting) induced glycogen depletion pre-slaughter is more likely to occur in SO and FOG fibres than in FG fibres in cattle and pigs. Thus darker muscles tend to be more prone to produce meat with high pH_u and dark cutting properties (Warriss, 1990; Wittmann et al., 1994; Fernandez et al., 1995; Warriss, 2000). Furthermore, Warriss et al. (1988) found that food withdrawal had no effect on glycogen content in the chicken *M. pectoralis superficialis* though it reduced the glycogen content in the *M. biceps femoris*. In addition, Henckel et al. (2000) found no difference in pH_u between *M. longissimus dorsi*, *M. biceps femoris*, *M. semimembranosus* and *M. psoas major* of well rested pigs. These studies suggest that environmental factors are more relevant in the induction of the frequently observed differences in glycogen content at the moment of slaughter or pH_u value between the different types of muscles rather than differences in physiological factors.

2.3.5 Other factors affecting muscle glycogen stores *pre mortem*

Besides the variation in genotype of the animals and the metabolic and contractile properties of different muscles, muscle glycogen content and subsequent meat quality are affected by several factors. These factors include pre-slaughter hormonal status, pre-slaughter stress (transportation, mixing of animals, lairage time, climatic factors), fasting, nutritional status, social and physical interactions as well as the physical fitness of the animal (Tarrant, 1981; Essén-Gustavsson et al., 1988; Warriss et al., 1989; Warriss, 1990; Fernandez & Tornberg, 1991; Essén-Gustavsson et al., 1992; Wittmann et al., 1994; Barton-Gade, 1997; Petersen et al., 1997; Henckel et al., 2002; Rosenvold & Andersen, 2003).

Fernandez and Tornberg (1991) reviewed the causes of variation in glycogen content of pig muscles and concluded that physical stress and handling of pigs from the farm to stunning (i.e. loading, waiting time in lairage, mixing, fasting) are probably the most potent factors lowering the glycogen levels in the live pig muscle. Pigs are very stress susceptible animals and even if handled in a way causing the animal minimal stress, the muscle glycogen content start to fall during the pre-slaughter period. Consequently, muscle glycogen content will be significantly lowered just prior to stunning (Fernandez et al., 2002; Henckel et al., 2002). Psychological stress, related to changes in physical and social environments, induces the release of catecholamines, which increase glycogenolysis (Monin, 2004). Furthermore, several workers have observed that in some pigs, glycogen undergoes an extremely rapid breakdown reaching low levels within a few hours after death (Briskey & Wismer-Pedersen, 1961; Sayre et al., 1963a; Kastenschmidt et al., 1968; Fernandez et al., 2002).

Sustained muscle exercise requires the mobilization of readily available muscle energy stores that are mainly glycogen. However, exercise before slaughter is not sufficient by itself to lower pig muscle glycogen content, but adrenalin treatment or adrenalin treatment combined with exercise does decrease muscle glycogen significantly (Henckel et al., 2002). Adrenalin administration also lowers muscle glycogen content in cattle (McVeigh & Tarrant, 1983; Tarrant & Lacourt, 1984; Immonen et al., 2000b). However, glycolysis in cattle is normally very aerobic. The average rate of glycogen breakdown in living muscle of young bulls was reported to be only 0.17 to 0.18 mmol/kg/min (with a maximum of 0.39 mmol/kg/min) despite severe stress from co-mingling or adrenalin administration (McVeigh & Tarrant, 1983; Tarrant & Lacourt, 1984). Nevertheless, fighting before slaughter is the major cause of depletion of glycogen stores in cattle and in the incidence of DFD meat (Warriss, 1987; Tarrant, 1989; Warriss, 1990; Grandin, 1993; Monin, 2004).

It is also possible to modify the muscle glycogen content of an animal during the rearing period. Porcine muscle glycogen content can be both increased and decreased by certain feeding regimes (Rosenvold & Andersen, 2003). Total glycogen content can be reduced through strategic finishing feeding (a diet with low content of digestible carbohydrates) without affecting the growth rate of the animals (Rosenvold et al., 2001; Rosenvold et al., 2003). The lower total glycogen content results from a reduction of macro-glycogen content (Rosenvold et al., 2003). However, as far as cattle are concerned, the diet is not a major determinant of muscle glycogen content and thus has only a minimal effect (Immonen et al., 2000a; Immonen et al., 2000b). Repeated moderate physical activity increases the resting

glycogen content in the muscles, which are involved during exercise in the pig (Essén-Gustavsson et al., 1988). However, Petersen, Henckel, Maribo, Oksbjerg and Sørensen (1997) reported that pigs reared in large pens (spontaneous activity) had less glycogen in their *M. biceps femoris* than individually penned or exercised pigs. Possibly this was due to a conversion of muscle fibres to the more oxidative type. However, it is generally believed that both endurance and sprint training increase the muscle's glycogen content in man (Saltin & Gollnick, 1983). Rearing pigs in cold ambient temperatures increases the glycolytic potential in FG muscle but decreases it in SO muscle (Lefaucheur et al., 1991).

A high glycogen content at the moment of slaughter may lead to a fast decrease in pH *post mortem* and a low pH_u, particularly in pigs and thus lead to a reduction in meat quality. On the other hand, low muscle glycogen content *pre mortem* may result in a limited decrease in pH, a high pH_u and also DFD formation. Thus, the optimal muscle glycogen content is somewhere between these two extremes.

2.4 The degradation of glycogen

2.4.1 The triggers of glycogenolysis

The rate of glycogenolysis in resting muscles is low. Phosphorylase, which is mainly responsible for the degradation of glycogen, is almost completely found in the low activity *b* form (Dalrymple et al., 1973; Chasiotis et al., 1982; Newsholme & Leech, 1983).

In contracting muscle, a glycogenolysis is triggered by the cascade of activation of glycogenolytic enzymes, which is initiated by an increased release of catecholamines (mainly adrenalin), an increase in the Ca^{2+} content in the cytosol, or by a combination of both processes acting in concert (Newsholme & Leech, 1983; Tarrant, 1989; Spriet et al., 1990). The catecholamines are released from neurones of the sympathetic nervous system (noradrenalin) and from the adrenal medulla (noradrenalin and adrenalin).

At an early stage of exercise, muscle contractions principally stimulate glycogenolysis (Richter et al., 1981; Richter et al., 1982). Contractions induce the conversion of phosphorylase *b* to the active phosphorylase *a* form through an allosteric activation caused by the release of Ca^{2+} from the sarcoplasmic reticulum (Roach, 2002; Nelson & Cox, 2005). This triggers an immediate glycogen breakdown. Continued glycogenolysis needs the direct effect of adrenalin on the muscle (Richter et al., 1982), otherwise phosphorylase soon reverts to resting levels even under conditions of continuous moderate intensity muscle stimulation (Conlee et al., 1979; Chasiotis et al., 1982; Richter et al., 1982; Ren et al., 1990; Hespel & Richter, 1992; Parolin et al., 1999).

Adrenalin is a very effective initiator of glycogenolysis. It mediates glycolysis through a series of biochemical changes that amplify the cyclic AMP-mediated pathway. In this way,

adrenalin activates vast amounts of phosphorylase extremely rapidly. A blood adrenalin content of only 10^{-10} M generates an increase in intracellular cAMP content to 10^{-6} M, and after several successive catalytic steps an amplification cascade of activated phosphorylase takes place (Lodish et al., 1995). The relative amounts of three successive enzymes in the glycogenolytic cascade; cAMP-dependent protein kinase, phosphorylase kinase and phosphorylase; occur as a 1:10:240 molar ratio in *M. psoas* (Cohen, 1978), indicating the extent of the amplification of the reaction chain. Hormonal mechanisms are also important for the mobilization and utilization of carbohydrates during stress (Roach, 2002; Nelson & Cox, 2005).

It has been suggested that muscle contraction may be the primary mechanism responsible for ante-mortem glycogenolysis in cattle (McVeigh & Tarrant, 1983; Tarrant, 1989) whereas increased catecholamine levels may be the predominant mechanism in pig and sheep (Monin et al., 1986; Tarrant, 1989). However, a fast *post mortem* glycogenolysis, particularly in the pig, is due to Ca^{2+} activation of myofibrillar ATPase (Greaser et al., 1969; Kastenschmidt, 1970). The ATP hydrolysis is probably characterized by the myofibrillar ATPases rather than the membrane ATPases also in muscle *post mortem* (Bendall, 1951; Bendall, 1973; Hamm, 1977).

2.4.2 Glycogen phosphorylase

Glycogen phosphorylase (EC 2.4.1.1) exists in two interconvertible forms, *a* and *b*. Phosphorylase *a* exists as both a tetramer and a dimer (Newgard et al., 1989), the most active form being the dimer (Fletterick & Madsen, 1980; Newgard et al., 1989; Nelson & Cox, 2005). Transformation of the *b* form to the active *a* form and the change in enzyme activity are controlled by three main stimulatory mechanisms: allosteric control by metabolite concentration during exercise, hormonal control by adrenalin secretion and nervous control by the release of Ca^{2+}.

Phosphorylase exists mainly in the *b* form in pig muscle at 10 min *post mortem* (Sayre et al., 1963a). Phosphorylase *b* is active in high AMP and inorganic phosphate (P_i) concentrations, but its activity decreases in high ATP and/or G-6-P concentrations (Newsholme & Start, 1979). Thus, in any situation in which the energy level of the fibre is decreased, or in which the G-6-P level is decreased, phosphorylase *b* will be activated and glycogenolysis will result. In contrast the phosphorylase *a* form is active in the absence of AMP but in the presence of ATP and G-6-P (Newgard et al., 1989). Muscular activity sharply but only temporarily increases the relative amounts of the *a* form (Chasiotis et al., 1982).

Glycolysis occurs mainly when ATP is dephosphorylated (Scopes, 1971). In stressful situations, phosphorylase *a* stimulates glycogenolysis by increasing the concentrations of the glycolytic intermediates in preparation for an increased demand for energy (Newsholme & Start, 1979; Richter et al., 1982). Despite the high rate of conversion of phosphorylase *b* to it's *a* form due to adrenalin stimulation, the rate of glycogenolysis remains low in non-contracting muscle and in resting muscle due to the low level of inorganic phosphate (P_i)

(Chasiotis et al., 1982; Fernandez et al., 1995). Furthermore, if the energy released by phosphorylase is not rapidly consumed, phosphorylase is inactivated and the hexose phosphates are reconverted to glycogen. In the event that the energy is utilized, AMP and phosphate further enhance the activation of phosphorylase. The rapid inactivation of phosphorylase is important, otherwise most of the glycogen would be degraded in about 10 seconds (Newsholme & Start, 1979).

Skeletal muscles contain much phosphorylase. The content of phosphorylase amounts to 2% of the total soluble protein in the muscle (Ryman & Whelan, 1971). There are about 20 to 25 phosphorylase tetramers or 40 to 50 dimers bound to each glycogen β particle (diameter of 40 nm), which can rapidly degrade glycogen at speed up to 30000 degradations particle^{-1} s^{-1} (Madsen & Cori, 1958). These glycogen particles (molecular mass 10^7 Da) comprise about 55000 glucose residues and about 2100 non-reducing ends (Goldsmith et al., 1982). High numbers of non-reducing ends enhance the capacity of phosphorylase molecules to bind to glycogen, as these correspond to increasing concentration of the substrate (Meléndez-Hevia et al., 1993). Thus, phosphorylase molecules work simultaneously on the many branches of glycogen, speeding the conversion of the polymer to monosaccharides.

A temperature of 39 ºC at a pH of around seven is optimal for the action of phosphorylase. The activity is sharply reduced by lower or higher temperatures (Cori et al., 1943; Schwägele et al., 1996). The most effective regulators of phosphorylase *b in vitro* are the substrate inorganic phosphate (P_i), the allosteric activators 5'-AMP and 5'-IMP, and the allosteric inhibitors ATP, ADP, and G-6-P (Cohen, 1978). P_i is one of the main factors determining phosphorylase activity and provides a link between the breakdown of PCr and glycogen utilization in muscle (Chasiotis et al., 1982). The activation, regulation, structure, kinetics and mechanism of phosphorylase have been dealt with comprehensively in several reviews (Fletterick & Madsen, 1980; Newgard et al., 1989; Johnson, 1992). For this reason, the regulation of phosphorylase activity will not be discussed any further.

Glycogen is optimally designed for the maximal action of phosphorylase under anaerobic glycolysis. In the presence of an excess of P_i, phosphorylase catalyzes the sequential phosphorolysis of the α-1,4-glycosidic linkages of glycogen yielding G-1-P and glycogen with shortened outer chains as products (Roach, 2002). Skeletal muscles contain plenty of phosphorylase, which is probably always saturated with its substrate glycogen, enabling a high rate of glycogen catabolism, and does not limit the rate of glycogenolysis even during high-intensity exercise (Shearer et al., 2001). However, phosphorylase alone is able to release a maximum of only 34.6% of the glucose units in the glycogen molecule (Meléndez-Hevia et al., 1993). Phosphorylase needs at least four-glucose monomers linked in a chain (A-chain) to act upon. Thus phosphorylase can act only on A-chains of glycogen (Meléndez-Hevia et al., 1993). A glycogen molecule maximally trimmed by phosphorylase in which the A-chains are four glucose units long is known as glycogen limited dextrin (or limit dextrin) (Walker & Whelan, 1960; Hers et al., 1964). The breakdown of the branching points of glucose chains and thereby the breakdown of glycogen limit dextrin is catalysed by the glycogen debranching enzyme (GDE) (Brown & Illingworth-Brown, 1966; Hers et al., 1967; Taylor & Whelan, 1968; Nelson et al., 1969).

2.4.3 Glycogen debranching enzyme (GDE)

2.4.3.1 The action of GDE

The glycogen debranching enzyme (EC 3.2.1.68) was found as an impurity in the preparations of rabbit muscle phosphorylase that caused an increase in the degree of phosphorolysis of glycogen over that of pure phosphorylase preparations (Cori & Larner, 1951). GDE is an indirect debranching system associated with the glycogen in mammals. It has the interesting property of having two separate catalytic activities associated within different domains on a single polypeptide chain (Brown & Illingworth, 1964; Brown & Illingworth-Brown, 1966; Nelson et al., 1970; White & Nelson, 1974; Bates et al., 1975; Gillard & Nelson, 1977) exhibiting maltooligosaccharide transferase (1,4-α-glucan:1,4-α-glucan 4-α-glycosyltransferase, EC 2.4.1.25) and amylo-1,6-glucosidase (dextrin 6-α-glucohydrolase, EC 3.2.1.33) activities. Both transferase and glucosidase activities of the enzyme are needed for complete glycogenolysis.

The substrate for GDE is the branch of glycogen which has been maximally trimmed by phosphorylase, i.e. a chain comprising four glucose units joined to each other by α-1,4- links before an α-1,6-link (A-chain). First, the maltooligosaccharide transferase (transferase) activity of GDE catalyses a transfer of a unit of three glucose residues from a side-chain to an adjacent α-1,4-glucosyl chain, leaving a single glucose unit attached by the α-1,6-link to the main chain (Figure 2). Either intermolecular or intramolecular transfer is possible (Brown & Illingworth, 1962; Brown & Illingworth, 1964). Second, the exposed single glucose residue is hydrolytically removed by α-1,6-glucosidase (glucosidase) activity of GDE (Brown & Illingworth, 1962; Abdullah & Whelan, 1963; Brown et al., 1963; Brown & Illingworth, 1964; Brown & Illingworth-Brown, 1966). Thus, the presence of the transferase together with the glucosidase will convert a substrate of one enzyme into a substrate for the other in tandem. The only low molecular weight product is glucose, and the net effect is an increase in the length of outer chains of glycogen but a decrease in number of the chains (Lee & Whelan, 1971). The total degradation of glycogen will cause 8% of the glucose stored in glycogen to be released in its free form by the action of GDE (Meléndez-Hevia et al., 1993), whereas the rest is released as G-1-P by the action of phosphorylase.

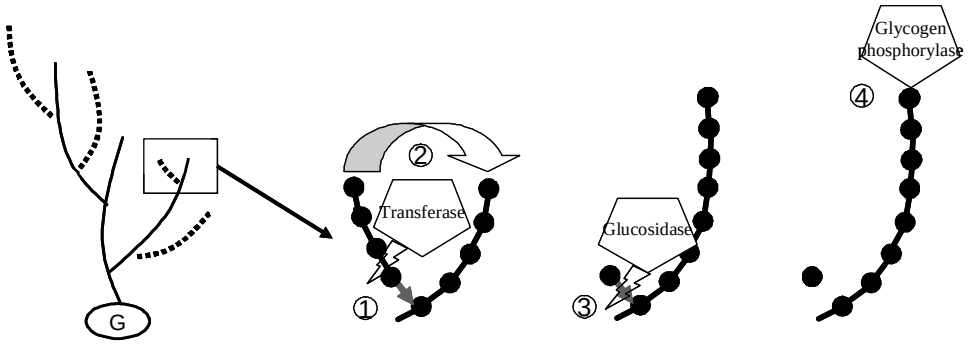

Figure 2. *The breakdown of glycogen limit dextrin by the glycogen debranching enzyme (GDE) according to Brown & Illingworth-Brown (1966). The maltooligosaccharide transferase activity of GDE catalyse two successive reactions: (1) a breakdown of an α-1,4-bond, (2) a transfer of three glucose units segment to an adjacent chain. (3) Amylo-1,6-glucosidase activity of GDE catalyses the hydrolysis of the exposed single α-1,6-bound glucose residue liberating a glucose unit. (4) The glucose chain is again susceptible the action of phosphorylase. An α-1,4-bond (—), an α-1,6-bond (—►), a glucose unit (●), glycogenin (G).*

The exact mechanism of the action of GDE is not clear. The pH optima of the two enzyme activities are different (Nelson et al., 1970), indicating two different catalytic sites on the enzyme. However, the polymer binding sites overlap or the sites strongly interact because they are flanked by a glucosidase site on one side and a transferase site on the other (Gillard & Nelson, 1977; Gillard et al., 1980; Takrama & Madsen, 1988; Liu et al., 1995; Nakayama et al., 2001). It has also been suggested that after the transferase reaction has taken place, the α-1,6-linked glucose unit rotates into the glucosidase catalytic site (Takrama & Madsen, 1988), or the completion of the transferase reaction may trigger a conformational change in the enzyme to create the glucosidase site (Liu et al., 1995).

It seems that GDE may not be active in muscle containing a relatively high content of normal structured glycogen (Nelson et al., 1972; Katz & Lee, 1988; Sahlin et al., 1989). Spriet et al. (1990) reported that the rate of glycogen breakdown was not altered in glycogen super-compensated, in normal or in fasted rat muscle even though exercise induced glycogenolysis accounted for over 50% reduction of the initial glycogen store. Nelson et al. (1972) showed that if the glycogen content is high, glycogen degradation is independent of the GDE in isolated protein-glycogen particles *in vitro*. On the other hand, both phosphorylase and GDE were active at lower glycogen levels. In exercising human muscle, glucose is formed by the action of GDE when the glycogen level decreases below 50 mmol/kg w.w. (Sahlin et al., 1989). Taylor et al. (1975) suggested that the activity of rabbit skeletal muscle GDE restricts G-1-P formation from glycogen limit dextrin to between 5 and 10% of maximal rate of phosphorylase.

It has also been suggested that degradation of glycogen by phosphorylase and GDE occur at the same time *in vivo*. Thus, a glycogen limit dextrin molecule, in which all the A-chains are four glucose units long, is never actually produced in the muscle of man under normal

physiological conditions (Newsholme & Leech, 1983). Nelson et al. (1972) suggested that the action of phosphorylase is random, which may explain results showing an absence of limit dextrin in a living fibre. After exposing a branching point of a glucose chain, phosphorylase moves to an adjacent chain. When the glycogen content is high, there are enough glucose chains in the glycogen molecules to constantly provide a substrate for phosphorylase. The action of GDE exposes the glucose chains to phosphorylase action again (Brown & Illingworth-Brown, 1966; Hers et al., 1967; Taylor & Whelan, 1968; Nelson et al., 1969), and by doing so it diminishes the total number of glucose chains. Thus, the importance of the GDE activity may increase with decreasing glycogen content. Furthermore, the intensity and duration of an exercise determines which one of the glycogen pools (pro-glycogen or macro-glycogen) is used for energy production. Thus exercise intensity and duration have an effect on substrate availability. Moreover, there could be differences in glycogen depletion patterns between species. It is conceivable that under certain conditions GDE might become a rate-limiting enzyme in glycogenolysis in living muscle and particularly in muscle *post mortem*. For instance, if the phosphorylase was in its active form, and the outermost chains of the polysaccharide had been removed.

2.4.3.2 Properties of the GDE

The GDE accounts for approximately 0.7% of the soluble muscle protein (Taylor et al., 1975; Becker et al., 1977). The monomer of GDE has a crescent or shrimp-like shape (Becker et al., 1977; Scraba et al., 1988). It has an estimated molecular weight of about 160 kDa (rabbit, chicken, porcine and human skeletal muscle GDE (White & Nelson, 1974; Taylor et al., 1975; Heizmann & Eppenberger, 1979; Scraba et al., 1988; Ding et al., 1990; Yang et al., 1992).

It seems that the GDE exists exclusively in an active state, since its activity is not altered by a variety of effectors involved in the control of glycogen metabolism such as: 5'-AMP, cAMP, ATP, ADP, IMP, UTP, ITP, UDPG, G-1-P, G-6-P, P_i or calcium ions (Brown & Illingworth-Brown, 1966; Becker et al., 1977). Moreover, covalently bound phosphate has not been detected in GDE (Becker et al., 1977). Thus, its activity appears to be quite independent of the mechanisms which activate phosphorylase *b* or which lead to phosphorylase *a* formation. However, phosphorylase and GDE regulate each other's capacity to act by means of substrate availability (Ryman & Whelan, 1971). The GDE has a high affinity for glycogen. The strength of binding of linear saccharides increases with chain length to a maximum of five glucose units. The binding also strengthens with the branching of substrate (Takrama & Madsen, 1988). Liu et al. (1995) found that saccharides containing one, two, or three glucose units are all competitive inhibitors of glucosidase activity, whereas all those with four or more units are non-competitive activators.

Gillard et al. (1980) found that glucosidase and combined transferase-glucosidase action are lost at the same rate when the GDE is exposed to the inhibitor, 1-S-dimethylarsino-1-thio-β-D-glucopyranoside, and suggested that the glucosidase activity of the enzyme is rate-limiting activity when it acts on glycogen limit dextrin. Brown and Illingworth-Brown (1966) reported that the transferase step is the slower of the two activities of GDE. This is supported

by other studies showing a faster rate of glucose production by GDE from α-glucosyl α-Schardinger dextrin than that from phosphorylase limit dextrin (Nelson et al., 1969; Tabata & Hizukuri, 1992).

2.4.3.3 The optimal conditions for the action of GDE

The issue regarding the pH optimum of GDE is confusing, and a number of conflicting pH optima values have been reported. The pH optimum for the release of glucose from glycogen limit dextrin varies widely from study to study. Extremes that have been reported are in the pH range of 5.0 to 6.4 (Hers et al., 1964) and 7.2 to 7.4 (Larner & Schliselfeld, 1956). Lee and Whelan (1971) have reviewed the results from different studies. One reason for the wide differences in pH values reported is that the pH optimum of GDE varies with the buffer used (Nelson et al., 1969; Nelson & Larner, 1970). The enzyme is less active when assayed in Tris buffer than with citrate buffer (Brown & Illingworth-Brown, 1966; Gordon et al., 1972). In addition, the shape of the pH optimum curve seems to be dependent on the buffer used. Taylor and Whelan (1968) reported a biphasic pH optima in citrate buffer, but a single optimum in citrate-phosphate buffer, However, Gordon et al. (1972) obtained a single pH optimum at about pH 6.0 for rabbit liver GDE both with citrate buffer and phosphate buffer. Dogfish GDE is inactive below pH 4.6 and above pH 9 with an optimum between the pH range of 5.5 and 6.3 in phosphate-citrate buffer (Becker et al., 1977).

The ambient temperature has a significant effect on the GDE activity. If the enzyme activity at 37 ºC and at its optimum pH range (pH 6.1 to 6.4) is taken as 100%, then it has 58% activity at 31 ºC and 38% at 25 ºC (Brown & Illingworth-Brown, 1966). Nelson and Watts (1974) reported a temperature optimum at approximately 50 °C for the rabbit muscle combined activities of GDE and separate glucosidase activity, whereas the temperature optimum of the transferase activity alone was lower, ca. 45 °C.

2.4.3.4 Measuring GDE activity

Several methods have been developed for measuring the GDE activity due to its property of having two separate catalytic activities (Brown & Illingworth, 1964; Brown & Illingworth-Brown, 1966; Nelson et al., 1970; White & Nelson, 1974; Bates et al., 1975; Gillard & Nelson, 1977). The two enzymatic activities of GDE can be assayed independently of each other or their combined action can be measured as one (Brown & Illingworth-Brown, 1966; Nelson et al., 1969; Nelson et al., 1970; Gillard & Nelson, 1977; Bollen & Stalmans, 1989).

The combined action of the GDE is assayed by measuring either the activity needed to release glucose from glycogen limit dextrin (Larner & Schliselfeld, 1956; Brown & Illingworth-Brown, 1966; Nelson et al., 1969; Gillard & Nelson, 1977; Lees et al., 2004) or by measuring the change in iodine staining capacity of glycogen limit dextrin (Nelson et al., 1970; Lee & Whelan, 1971). Hers et al. (1967) obtained similar results with both methods. The GDE complex elongates the outer chains of limit dextrin resulting in the formation of a

more ‘glycogen like’ structure, which can be determined as a shift in the iodine complex spectrum of limit dextrin. Glycogen limit dextrin is the best substrate for the combined activity assay of the enzyme in rabbit skeletal muscle. This is because the enzyme acts at a much slower rate with native glycogen, amylopectin and many artificial substrates (Nelson et al., 1969; Lee & Whelan, 1971; Liu et al., 1991).

The transferase can catalyze the elongation of the outermost tier chains of amylopectin, glycogen and limit dextrin (Nelson et al., 1970), which are the main substrates of this enzyme’s activity. Glucose, maltose, maltotriose, maltotetrose and maltopentose can all act as acceptors of maltosyl or maltotriosyl units transferred from suitable donors but none of these five compounds can function as a donor in the transferase reaction (Brown & Illingworth, 1964) because the donor has to be six or more glucose units long. Glucose and the series maltose to maltopentose were found to be acceptors of glucosyl segments transferred from glycogen but they were not altered when incubated alone with the enzyme (Lee & Whelan, 1971). In contrast, maltohexose and higher oligosaccharides acted as both donors and acceptors of maltosyl and maltotriosyl units (Lee & Whelan, 1971). α-maltotriosyl fluoride (Braun & Withers, 1995) and 6^3-α-maltotriosylmaltotetraose (B_7) (Brown & Illingworth-Brown, 1966) are specific substrates developed for the measurement of transferase activity in purified preparations. The transferase activity can also be assayed by measuring the change in iodine staining capacity of amylopectin (Nelson et al., 1970; Lee & Whelan, 1971).

Glucosidase specifically hydrolyses the α-1,6-link between glucose units of an oligosaccharide. It can also hydrolyse the α-1,6-link of a polysaccharide in which the outer branch point glucose units are exposed, and which has its main chains in spatial association with one or more glucose residues beyond the units involved in branching (Brown & Illingworth-Brown, 1966). The glucosidase step is slightly reversible since it can also catalyse the incorporation of ^{14}C-glucose into polysaccharides such as glycogen (Hers & van Hoof, 1966; Hers et al., 1967; Nelson & Larner, 1970; Taylor et al., 1975; Gillard & Nelson, 1977; Heizmann & Eppenberger, 1979). Furthermore, specific substrates have been developed for the separate measurement of glucosidase activity. Glucosidase is able to release glucose from 6^3-α-glucosylmaltotetraose (fast B_5) (Brown & Illingworth, 1962; Brown & Illingworth-Brown, 1966), α-D-glucosyl fluoride, and from several branched cyclodextrins (Taylor & Whelan, 1966; Tabata & Hizukuri, 1992; Liu et al., 1995). The most specific substrate is 6-O-α-D-glycosyl-α-cyclodextrin (α-Schardinger dextrin). However, its susceptibility is lower than that of glycogen phosphorylase limit dextrin (combined action of GDE) and 6^3-α-glucosylmaltotetraose (Omichi & Hase, 1998). These more susceptible substrates are, also hydrolysed to produce glucose by the actions of other enzymes such as α-amylase (EC 3.2.1.1) and lysosomal α-glucosidase (EC 3.2.1.20), the combined effects of which result in an overestimation of GDE activity in raw meat extracts and impure enzyme preparations (Lee & Whelan, 1971; Omichi & Hase, 1998). The incorporation of ^{14}C-glucose in glycogen can be used for measuring both the GDE activity of raw meat extract and the activity of purified enzyme (Nelson & Larner, 1970), but this method also overestimates the GDE activity. One cause of the overestimation is that radioactive glycogen of lower molecular weight is formed from the mother glycogen by the transglycosylation action of endo-hydrolytic enzymes (Omichi & Hase, 1998).

Sample preparation and storage have an influence on GDE activity. Freezing and subsequent frozen storage causes a loss of GDE activity in purified preparations (Nelson & Larner, 1970; Nelson & Watts, 1974). The extent of loss depends on the buffer in which the enzyme is frozen (Nelson & Watts, 1974). Moreover, dilution leads to a loss of activity, but the activity is regained by incubation in the presence of an inert protein (gelatin) (Nelson & Watts, 1974). However, the rate of action of the GDE is slower when it is bound to a protein-glycogen particle than its rate of action either as a purified enzyme or when the particle is dissociated by dilution (Nelson et al., 1972).

As stated above, several methods have been developed, where the combined GDE activity is measured in a raw meat extract or as the purified enzyme. It is not completely clear which of the activities of the GDE is the rate-limiting step. By measuring the combined activity of the enzyme this question can be circumvented. Furthermore, it is not even relevant which one of the activities is rate-limiting when the pH decrease of meat is in focus.

The liberation of free glucose from glycogen limit dextrin should perhaps be a more accurate method for measuring the combined activity of GDE than the modification of the iodine spectrum. However, the former method is subject to interference by non-specific glucosidases when using a crude tissue extract as a source of the enzyme (Hers et al., 1967). Apart from amylo-1,6-glucosidase, muscles also contain other glucosidases such as acid α-glucosidase in lysosomes (Roach, 2002), acid phosphatases and maybe also amylase activity. At least the latter is capable of the slow digestion of glycogen to low molecular weight oligosaccharides (Meyer et al., 1970). The action of GDE on limit dextrin changes the resulting iodine-complex spectrum, and this change is dependent on both glucosidase and transferase action when a tissue extract is used as the source of GDE (Nelson et al., 1970). Furthermore, the change in iodine spectrum is specific for GDE activity, as α-glucosidase is not capable of producing similar changes in iodine staining capacity of limit dextrin. In addition, one should be aware that saccharides with a chain length of four glucose units or greater are bound differently and tighter to the enzyme than shorter molecules (Liu et al., 1995). Thus, the use of artificial substrates for the enzyme activity assay may result in an underestimation of the actual GDE activity.

2.5 Ultimate pH and residual glycogen content

The extent of the decrease in pH in muscle *post mortem,* mainly depends on the energy stores available in the muscle at the time of slaughter (Briskey & Lawrie, 1961; Bendall, 1973; Hamm, 1977; Warriss et al., 1989), and the buffering capacity of the muscle (Bate-Smith, 1938; Davey, 1960; Lykkeboe & Johansen, 1975; Talmant et al., 1986; Rao & Gault, 1989; Kivikari, 1996; Kylä-Puhju et al., 2004). The buffering capacity, which depends on the animal species and the type of muscle, varies between 40 to 60 mmol LA equivalent/(kg*pH) in pig, cattle and chicken (Kivikari, 1996; Kylä-Puhju et al., 2004). However, lower and higher values have been reported as reviewed by Kivikari (1996). The extent of the decrease in pH is smaller in poultry, where pH_u is normally ≥ 5.7 compared with the range of 5.4 to 5.7 in most muscles of other meat producing species (Monin, 2004).

The amount of glycogen determines the extent of the pH decrease, but only if the glycogen content has decreased below a critical value. Thus, the level of GP or glycogen content is not the main factor determining the pH_u in the muscles of well-fed and rested animals. Variation in GP accounts for a maximum of 50% of the differences in the pH_u values of pork loin (Maribo et al., 1999; van Laack & Kauffman, 1999) and in the *M. adductor* (Warriss et al., 1989). The pH_u follow a negative curvilinear dependence on GP until GP reaches a convergence point or threshold value. Further increases in glycogen content beyond this threshold value, does not have an influence on pH_u (Bendall, 1973; Fernandez & Gueblez, 1992; Przybylski et al., 1994; Wittmann et al., 1994; Immonen & Puolanne, 2000; Henckel et al., 2002). This threshold value is 53 mmol/kg glycogen at stunning for the porcine *M. longissimus dorsi* (Henckel et al., 2002) and 57 mmol/kg for bovine muscles (Tarrant, 1989). If the glycogen content in muscles before slaughter is high, i.e. above the threshold value (Lawrie, 1955; Bendall, 1973; Przybylski et al., 1994; Henckel et al., 2002), the glycolysis in mammalian muscles ceases when the pH falls to ultimate values between 5.3 to 6.3 depending on the animal species and the type of muscle (Briskey & Lawrie, 1961; Newbold & Harris, 1972; Bendall & Swatland, 1988; Rao & Gault, 1989; Fernandez & Tornberg, 1991; Honikel, 1992; Fischer & Dobrowolski, 2002). Ultimate pH usually remains higher in SO than in FG muscles (Beecher et al., 1965b; Bendall, 1975; Laborde et al., 1985; Bendall & Swatland, 1988; Karlsson et al., 1993).

It has been suggested that various factors influence pH_u when glycogen is not the limiting factor. These include initial metabolite concentrations of PCr, ATP, and lactate (Kastenschmidt et al., 1968; Lundberg et al., 1987; Pearson & Young, 1989a; Rhoades et al., 2005). *Post mortem* glycolysis may be stopped by AMP deficiency (Scopes, 1971), a shortage of ADP or of glucose (van Laack et al., 2001) and an inhibition of glycolytic enzymes by pH decrease (Lundberg et al., 1987; Pearson & Young, 1989a). Kastenschmidt et al. (1968) and Rhoades et al. (2005) suggested that glycolysis was stopped because of an inhibition of the action of 6-phosphofructokinase by a low pH. The subsequent increase in G-6-P content led to the inhibition of phosphorylase. Furthermore, Fischer and Dobrowolski (2002) reported a range between 0.4 to 9.9 mmol/kg of G-6-P in different porcine muscles 24 h after slaughter. The highest G-6-P contents were found in muscles, which had the lowest pH_u values and the highest residual glycogen contents. Moreover fast chilling in some cases results in higher pH_u values of meat as reviewed by Pösö and Puolanne (2005).

Several studies have shown that some glycogen always remains unconverted to lactate in muscles after the *post mortem* reaction sequence ceases (Lawrie, 1955; Lawrie et al., 1959; Monin et al., 1987; van Laack & Kauffman, 1999). Immonen and Puolanne (2000) showed that in bovine muscles with pH_u lower than 5.75 the residual glycogen content varies considerably, from 10 to 85 mmol/kg meat. RN^- gene carrier pigs have higher initial and residual glycogen contents but lower pH_u values compared to wild type pigs (Enfält et al., 1997a; Lundström et al., 1998). The organization and size of the different glycogen particles is potentially of considerable importance to their metabolism. Lawrie et al. (1959) reported that the residual glycogen of the *M. sternocephalicus* had shorter external chain lengths than of the *M. psoas major*, which attained lower pH_u values. Moreover, Briskey and Lawrie (1961) reported that glycogen samples isolated from different bovine muscles during *pre*

rigor and *post rigor* were broken down at unequal rates by phosphorylase and the rate of breakdown seemed to be somewhat related to glycogen chain length. Thus, GDE may have a role in determining pH_u of meat.

2.6 The significance of pH decrease after slaughter

Both the rate and the extent of the *post mortem* decrease in pH determine the palatability of meat by affecting several meat quality properties including: drip loss, colour development, shelf life, water-holding capacity, texture, tenderness and eating quality in general. These properties have been covered in detail in several reviews (Briskey, 1964; Bendall, 1973; Hamm, 1974; Asghar & Pearson, 1980; Seideman et al., 1984; Bendall & Swatland, 1988; Offer & Knight, 1988a; Offer & Knight, 1988b; Pearson & Young, 1989a; Fernandez & Tornberg, 1991; Warner et al., 2001).

A high pH_u value is associated with better water-holding capacity, higher processing yield and redder colour, but the flavour of fresh meat and its shelf life may be compromised. Thus, when the technological and economic quality criteria of pork are concerned, the higher the pH_u is the better is its quality (Warriss & Brown, 1987). However, there are exceptions to this: dark, firm and dry (DFD) meat occurs as a result of a limited pH decrease and is undesirable (Bendall & Swatland, 1988; Offer & Knight, 1988b; Pearson & Young, 1989a; Monin, 2004). A decrease in meat quality may also occur, if the pH_u of the meat is too low (acid meat) or if the rate of pH decline had been high (Bendall & Swatland, 1988; Sellier & Monin, 1994; Monin, 2004). In normal muscle, ATP and PCr decrease slowly and P_i increases steadily, whereas in PSE prone muscle, ATP and PCr are quickly exhausted (Kastenschmidt et al., 1968; Miri et al., 1992). The development of PSE is the result of an increased glycolytic rate and rapid decrease in pH early *post mortem*, when the muscles are still warm (Bendall & Wismer-Pedersen, 1962; Fischer et al., 1979; Bendall & Swatland, 1988; Offer, 1991; van Laack et al., 2000). The development of PSE may occur through excessively rapid glycolysis, a particularly low pH_u, slow chilling, or various combinations of these factors (Offer & Knight, 1988b; Offer, 1991; van Laack et al., 2000; Monin, 2004; Sams & Alvarado, 2004) and leads to the denaturation of proteins with a great impact on meat palatability (Asghar & Pearson, 1980; Offer, 1991; Warner et al., 1997; Joo et al., 1999; Monin, 2004).

The basic biochemical reactions underlying the pH decline *post mortem* and how this decline exerts a strong influence on a number of important meat quality aspects are well recognised. Animal breeding, however, has mainly focused on increasing the growth rate and the muscle mass of meat production animals. Thus, it seems that the anaerobic glycolysis plays an increasing role in energy production of a living muscle. Profound knowledge about glycogen degradation in both *pre mortem* and *post mortem* muscles is needed for that the final quality of meat can be controlled.

3 OBJECTIVES OF THE STUDY

The goal of this work was to investigate the role of glycogen debranching enzyme (GDE) in glycolysis in the muscles of meat production animals and also its role in the attainment of the ultimate pH (pH_u) of meat.

The objectives of the present study were to investigate:

1. The effects of pH and temperature on GDE activity (Study I).

2. The GDE activity in porcine, bovine and poultry muscles (Studies II and III).

3. The relationships between GDE and phosphorylase activities in slow twitch oxidative muscles and in fast twitch glycolytic muscles (Studies II and III).

4. The relationship between GDE activity and pH_u and the stability of the GDE activity after slaughter (Studies II, III and IV).

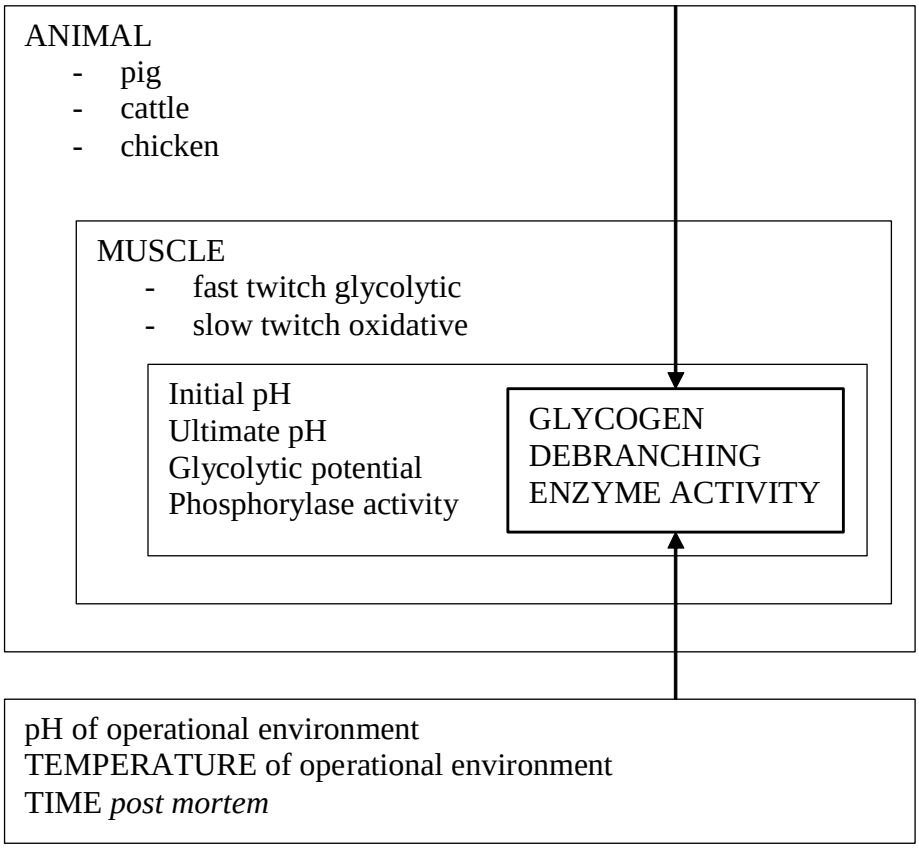

Figure 3. *The set of variables.*

4 MATERIALS AND METHODS

The materials and methods used in this work are described in detail in the original papers Studies I, II, III and IV.

4.1 Animals and sampling

Muscle samples were obtained from commercial abattoirs. For Study I, samples were collected from 12 pigs (*M. longissimus dorsi, M. masseter*). For Study II samples were collected from 19 bovines (*M. longissimus dorsi, M. masseter*) and 27 pigs (*M. infraspinatus, M. longissimus dorsi, M. masseter, M. semimembranosus*). For Study III, samples were collected from 25 chickens (*M. pectoralis superficialis, M. quadriceps femoris*). For Study IV, samples were collected from 20 pigs (*M. longissimus dorsi, M. semimembranosus*). The samples were taken about 35 min (porcine and bovine muscles) or about 25 min (chicken muscles) after stunning. Samples, except the samples for ultimate pH analysis, were diced and frozen immediately in liquid nitrogen and stored at -80 °C until analysis. For ultimate pH value measurements the samples were kept at 4 ºC for 24 h (porcine and chicken muscles) or 48 h (bovine muscles) *post mortem* before freezing (-80 ºC).

4.2 Biochemical and physical methods

Each sample was assayed for glycogen debranching enzyme (GDE) activity, total glucose content, lactate content and pH value. Most of the samples were also analysed for phosphorylase activity (Studies I, II and III), and a few samples analysed for the transcription level determination of GDE gene (Study IV).

4.2.1 The glycogen debranching enzyme activity

The GDE activity was measured by a method that determines the change in the iodine complex spectrum of phosphorylase limit dextrin (Nelson et al., 1970). The measurements were designed for measuring the GDE activity under conditions resembling intact muscle. The method enables the use of crude meat extract and the natural limit dextrin substrate in the GDE activity analyses. Glycogen limit dextrin is not commercially available. It was produced by the exhaustive action of purified phosphorylase on glycogen according to Werries et al. (1990) with small modifications. Commercial phosphorylase *a* may contain traces of GDE, therefore, purification is a prerequisite for the successful preparation of limit dextrin.

Purification of glycogen phosphorylase

Phosphorylase *a* was purified chromatographically with ω-aminobutyl agarose matrix at 4°C (Chen et al., 1987). The matrix was regenerated with 0.1 M acetic acid (pH 5.0) and equilibrated with 5 mM Tris buffer (pH 7.2) containing 1 mM EDTA and 14 mM 2-mercaptoethanol (equilibration buffer). Phosphorylase *a* (rabbit muscle, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in equilibration buffer and subsequently pipetted into a column (Pharmacia Fine chemicals, Uppsala, Sweden). After equilibration, phosphorylase *a* was eluated from the column with equilibration buffer containing 250 mM NaCl. The fraction containing purified phosphorylase *a* was dialysed in distilled H_2O at 4°C overnight. It was subsequently frozen and freeze-dried. The activity of purified phosphorylase *a* was checked (see method description below).

Preparation of glycogen limit dextrin

Purified phosphorylase *a* was dissolved in 5 ml 0.05 M phosphate buffer (pH 6.8) containing 300 mg glycogen (bovine liver) and 10 µl 10 mM 5'AMP. The solution was dialysed at 37 °C in 80 ml of phosphate buffer for 24 h. The dialysis solution was changed once during this period. After dialysis, phosphorylase *a* was inactivated by tricloroacetic acid. The solution was dialysed in distilled water overnight. The water was changed once during this period. The dialysed solution was centrifuged (at 2400 rpm for 10 min) at 4 °C and the limit dextrin in the supernatant was precipitated with ethanol (1:4 dilution) added as four aliquots. The solution was kept at +4 °C and centrifuged (at 2400 rpm for 5 min) the resulting pellet was washed twice with ethanol and centrifuged again (at 2400 rpm for 5 min). The washed pellet was vacuum dried at -86 °C overnight.

The produced limit dextrin may not have been completely homogenous and separate lots of limit dextrin were pooled before starting the analyses. Thus, despite the possibility that the produced limit dextrin was not completely homogenous, the measured enzyme activities are comparable.

Measuring the glycogen debranching enzyme activity

The combined GDE activity was measured by a colorimetric assay based on a shift in the absorbance at 525 nm upon GDE-catalyzed conversion of limit dextrin to glycogen (Nelson et al., 1970). The reaction mixture (pH 6.3±0.05) contained 0.1 ml 1% limit dextrin and 0.020 ml 0.5 M sodium maleate. The reaction mixture was heated in a water bath to 39 °C and the reaction started by adding 0.08 ml meat extract. The meat extract was prepared daily, using 2.5 ml buffer that contained 0.05% $KHCO_3$ and 4 mM EDTA (pH approx. 7.8 at 25 °C) per 1 g muscle (w.w.). The mixture was homogenised (Ultra-Turrax T25, Ika-Werke GMBH, Staufen, Germany) and centrifuged (15840 rpm for 10 min) at 10 °C (Sorvall Instruments RC5C, Du Pont Company, Wilmington, USA, equipped with SS34 rotor). The supernatant that was obtained was used for the measurements.

After certain reaction times the GDE catalyzed reaction was stopped by immersing the reaction mixture in its container into a boiling-water bath and subsequently by immersing the same in an ice bath. Preliminary experiments were conducted to establish the suitable reaction times to fit the time points in the linear phase of the curve. Each animal species and each muscle were studied separately. The reaction times were: 1, 1.5, 2.5 min for porcine

M. longissimus dorsi; 1, 2.5, 4.0 min for porcine *M. infraspinatus* and *M. masseter*; 1, 2, 3 min for porcine *M. semimembranosus*; 1, 3, 6 min for bovine muscles and 1, 4, 8 min for chicken muscles.

Iodine reagent (2.6 ml) was added to the stopped reaction mixture then the whole was allowed to equilibrate for 20 min. The mixtures were filtered into cuvettes through cotton wool to prevent the developed deposit to get involved and the absorbances (525 nm) of the mixtures were recorded. The iodine reagent was prepared by dissolving 0.26 g of I_2 and 2.6 g KI in 10 ml distilled water as described by Nelson et al. (1970). The 1.0 ml of the I_2-KI solution was added to 260 ml of saturated $CaCl_2$ solution (pH 5.6 to 6.2 at 1 to 10 dilution) to produce the iodine reagent.

In addition, the absorption spectra between 375 nm and 800 nm of the blank and the reaction mixtures were obtained to ensure the conversion of limit dextrin to glycogen. The assay measures the combined GDE activity, i.e. both glycan transferase (EC 2.4.1.25) and amylo-1,6-glucosidase (EC 3.2.1.33) activities. The GDE elongates the outer chains of limit dextrin resulting in the formation of a more 'glycogen like' structure (Nelson et al., 1970). This was observed as a shift in the absorption spectra of limit dextrin to that more closely resembling the absorption spectra of glycogen (Figure 4). Even so the spectra did not entirely match the absorption spectra of glycogen, indicating that substrate availability was not a limiting factor, i.e. the reaction had the potential to continue at maximum velocity.

The GDE activities were assayed in triplicate for each of three separate time points, and the activity was calculated from the slope of the linear phase of the absorbance curve. Method blanks (zero time controls) were prepared by denaturing the meat extract protein. This was accomplished by immersing a tube containing the meat extract in a boiling-water bath before adding the other reagents. For the determination of the temperature-activity curves and pH-activity curves (Study I), the GDE activity was measured at temperatures 5, 15, 25, 35, 39, 42, 50, 60 °C (at pH 6.3±0.05) and in pH values 7.2, 6.8, 6.4, 6.3, 6.2, 6.0, 5.6, 5.0 (at temperature 39±1 °C), respectively. When the GDE activity was measured in different muscles, the pH was adjusted to 6.3±0.05 and the temperature to 39±1 °C according the maximum GDE activity obtained from the temperature-activity profiles.

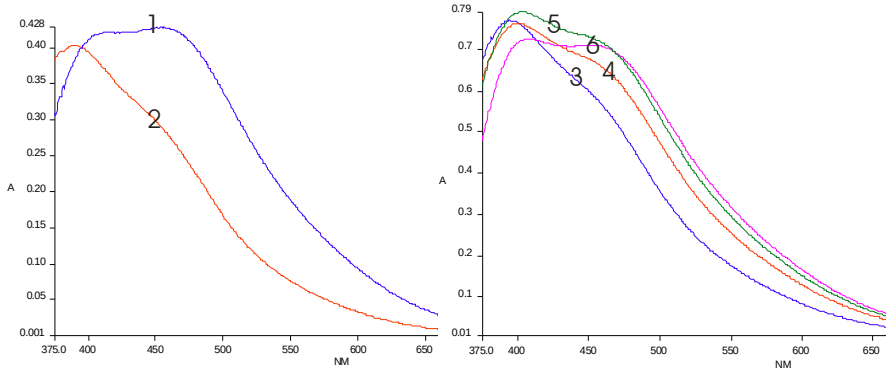

Figure 4. *The absorption spectra of glycogen (1), glycogen limit dextrin (2), and the change in the spectrum of glycogen limit dextrin during the GDE catalyzed reaction (porcine M. longissimus dorsi): blank (3), at 1 minute reaction time (4), 1.5 minute reaction time (5), and 2.5 minute reaction time (6).*

Some experiments were performed by substituting Na-maleate buffer with citrate-phosphate buffer (n=3) and the GDE activity was also measured in the presence of urea (1 M) (n=3) to determine the influences on the pH-GDE activity curves.

4.2.2 Total glucose, lactate, pro-glycogen and macro-glycogen contents

For total glucose and lactate determinations, muscle samples were homogenised in ice-cold 0.1 M phosphate buffer (pH 7.0) with a Polytron homogeniser (Thomas Scientific, USA). Total glucose content (glycogen, glucose and G-6-P) was determined by hydrolysing 10 µl of the homogenate in 0.1 M HCl (at 100 °C, for 2 h), after which the pH was adjusted to between 6.5 and 7.5 (Lowry & Passoneau, 1973). Glucose was determined with a Roche diagnostic kit no. 1447521. The lactate content was determined from the same homogenate spectrophotometrically (365 nm) using Boeringer-Mannheim Diagnostic Kit no. 139 084.

Pro-glycogen and macro-glycogen (Study IV) were separated by precipitation in perchloric acid (Adamo & Graham, 1998). Both glycogen fractions were analysed separately as described above. The free glucose and G-6-P were included in the macro-glycogen fraction and thus glycogen, glucose and G-6-P, all contribute to the total glucose amount.

The glycolytic potential (GP) is a measure of all the compounds present in the muscle that can be converted into lactate, thus GP is an index of the muscle's capacity for glycolysis *post mortem*. GP (mmol LA equiv./kg) can be calculated as a sum 2([glycogen] + [glucose] + [G-6-P]) + [lactate] (Monin & Sellier, 1985). The GP value remains constant after slaughter, only the ratios between the metabolites constituting GP change (Maribo et al., 1999).

4.2.3 Phosphorylase activity

The phosphorylase activity was measured spectrophotometrically (340 nm) by following the release of G-1-P from glycogen (Bass et al., 1969). The muscle sample (0.3 g) was homogenized in 3 ml of 50 mM Tris-HCl buffer (pH 7.6) and diluted (1:5) with distilled water. The reaction mixture (total of 2.5 ml) contained 50 mM Tris (pH 7.6), 5 mM EDTA, 10 mM KH_2PO_4/Na_2HPO_4 (pH 7.6), 10 mM L-cystein, 0.3 mM NADP, 0.05 mM glucose-1,6-diphosphate, 2 mM AMP, 1.68 U phosphoglucomutase, 28 U glucose-6-phosphate dehydrogenase and 0.092 ml of diluted sample. The reaction was started by adding 0.5 g glycogen and the slope of increase in absorbance was determined. The assay measures the combined maximum activity of phosphorylase (*a* + *b*) (Bass et al., 1969).

4.2.4 The transcription level of GDE gene

The transcription levels of GDE gene (Study IV) and a housekeeping gene β-Actin were quantified with real time RT-PCR. The RNA was purified using the method of Chomczynski and Mackey (1995). Total RNA was determined by measuring the absorbance at 260 nm. Equal amounts of RNA were reverse transcribed as described by Theil et al. (2006). Reverse transcribed material was amplified using primers specific for each gene. Primers and probes were designed by using Primer Express version 2.0 software and either a primer or a probe was designed to anneal to a splice site to avoid amplification of genomic DNA. The signal was detected quantitatively by gene specific probes labelled with FAM^{TM} fluorophore at the 5'end.

4.2.5 The pH value

In Studies I, II and III, the pH values were measured by homogenising frozen samples (1 g) in ice cold 5 mM Na-iodoacetic acid (10 ml) and the pH value of the extract was measured (Knick Portamess 752 pH-meter, Mettler-Toledo Inlab 427 electrode) at room temperature. The pH electrode was calibrated in pH 4.01 and 7.00 buffers at ambient temperature. In Study IV, the pH was measured (Knick portable pH-meter, combination gel electrode, SE104, Knick Berlin, Germany) in *M. longissimus dorsi* at the last thoracic vertebra and in the middle of *M. semimembranosus* at 0.5, 3, 5, 24 and 48 hours after bleeding. The pH electrode was calibrated in pH 4.01 and 7.00 buffers at ambient temperature when pH was measured 30 min *post mortem*, and at 4°C at the other time points.

4.3 Statistical analysis

The data were analyzed applying standard statistical procedures as described in the original papers (I, II, III and IV). The statistical programs used in the analyses were SAS v8.02 and SPSS 10.0 for Windows.

Pearson's correlation coefficient was used to test the correlations between the variables (GDE activity, phosphorylase activity, GP, pH_{35} and pH_u) within a muscle (Study II) and partial correlations were calculated by correcting for the day of slaughter as an environmental variable (Study III). Regression analysis was used to obtain the parameter estimates in temperature-activity curves and in pH-activity curves (Study I) and the activity curves were fitted with SAS/insight. Regression analysis used the day of slaughter as a factor to compensate for environmental effects when explaining variation in pH_u (Study III).

The independent samples t-test was applied to data from the chicken samples, to test the difference in GDE activity, phosphorylase activity and the PHOS:GDE ratio between low and high GDE groups within a muscle (Study III). In addition, the independent samples t-test was used to analyse the differences in same variables between bovine muscles (Study II).

The General Linear Model including muscle as a fixed effect and slaughter date as the random effect was applied when calculating the estimated marginal means for GDE activity, phosphorylase activity and PHOS:GDE ratio in porcine and chicken muscles (Studies II and III). The General Linear Model was also used when comparing variables among porcine, bovine and chicken muscles. The differences in transcription levels of GDE between RN genotypes were also tested by using the GLM procedure (Study IV). The MIXED procedure used factors including genotype, sex, time and combined effect of genotype and time as fixed factors and individual animal as a random factor and it was used when testing the differences between different RN genotypes and sampling times in GDE activity, carbohydrate compounds and also pH values (Study IV).

5 RESULTS

5.1 The effect of temperature and pH on GDE activity

The effect of temperature and pH on the GDE activity was studied in porcine muscles (Study I). The optimum temperature for GDE in *M. longissimus dorsi* was around 40 °C, but higher in *M. masseter*, i.e. between 40 °C and 50 °C. The GDE activity decreased with temperature from 42 °C to 4 °C in both muscles. The difference in GDE activity was very highly significant ($P<0.001$) between carcass temperatures during cooling (4 °C, 15 °C) and the carcass temperatures just after slaughter (39 °C, 42 °C). In *M. masseter*, the GDE activity was already lower at 25 °C than at 39 °C ($P<0.01$). The activity was almost zero when the temperature decreased below 15 °C in *M. longissimus dorsi* and below 25 °C in *M. masseter*.

In both muscles, the GDE was active over the pH range of 5.0 to 7.4. A slight maximum activity peak for GDE was detected around pH 6.5 for the *M. longissimus dorsi*. On the other hand, the GDE activity increased slowly with decreasing pH and no relative maximum peak for the GDE activity could be found within the pH range studied for the *M. masseter*. Hence,

pH had only a weak effect on the GDE activity in either muscle over the pH range found in carcasses post-slaughter.

The change of maleate buffer to citrate-phosphate buffer or the presence of 1 M urea in the reaction mixture had no significant effect on the pH-activity curve of GDE either in the *M. longissimus dorsi* or in the *M. masseter* (preliminary experiments).

5.2 GDE activity in the muscles of meat producing animals

The GDE activity was studied in fast twitch glycolytic (FG) and slow twitch oxidative (SO) muscles of pig, cattle and chicken (Studies II and III). The differences in GDE activity between the animals (Table 1) were most pronounced in the FG muscles. The GDE activity was: highest in the porcine *M. longissimus dorsi*, intermediate in the bovine *M. longissimus dorsi* and lowest in the chicken *M. pectoralis superficialis* ($P<0.001$). The differences between the SO muscles of the studied animals were less evident. The GDE activity was again highest ($P<0.001$) in the porcine *M. masseter*, but similar between the bovine *M. masseter* and the chicken *M. quadriceps femoris.*

Table 1. *The GDE activity (Δabs/Δmin) in porcine, bovine and avian muscles.*

	MUSCLE TYPE	PORCINE N=27	BOVINE N=19	AVIAN N=25
M. infraspinatus	SO	0.089b		
M. masseter	SO	0.073a	0.047a	
M. longissimus dorsi	FG	0.187c	0.091b	
M. semimembranosus	FG	0.160c		
M. quadriceps femoris	SO			0.048b
M. pectoralis superficialis	FG			0.014a
S.E.		0.006	0.004	0.002

S.E. = standard error of the mean within a column. Different letter within a column denotes a significant difference between the values.

When the muscle types were compared the GDE activity was about twice as high in porcine and bovine FG muscles than in SO muscles. The differences between the porcine FG and SO muscles were also clearly seen in Study I. On the other hand, differences in the GDE activity between the muscles of the chicken were exactly the opposite to that found in red meat species. In FG *M. pectoralis superficialis* the GDE activity was only a third of the activity of that in SO *M. quadriceps femoris* (Table 1).

5.2.1 Phosphorylase activity and the ratio between phosphorylase and GDE activities in different muscles

The phosphorylase activity was higher in the FG muscles than in the SO muscles of pig, cattle and chicken (Studies II and III). Of the species studied the phosphorylase activity was highest in chicken muscles: 18.0±5.8 U/g muscle in *M. pectoralis superficialis* and 5.9±2.7 U/g muscle in *M. quadriceps femoris*. In contrast, phosphorylase activity was lowest in bovine muscles: 8.0±1.5 U/g muscle in *M. longissimus dorsi*, 1.3±0.4 U/g muscle in *M. masseter*. A significant correlation between the GDE activity and the phosphorylase activity was found in only one muscle, porcine *M. infraspinatus* ($r = 0.426$, $P = 0.027$).

The ratio between phosphorylase activity and GDE activity (PHOS:GDE) was calculated for different muscles from their respective activities of both glycogen degrading enzymes. The aim was to obtain information about the relationships between these enzymes. However, phosphorylase and GDE activities were not measured in the same units, hence the ratio does not quantify the true difference between the activities of these enzymes within a muscle. Nevertheless, the ratios are relative values and can therefore be used to make comparisons between muscles. The values for the PHOS:GDE ratios were higher in the FG muscles than in the SO muscles. The value for the ratio (with standard deviation) was 46±17 in SO porcine muscles and 74±25 in porcine FG muscles. In the bovine, the ratio was 30±10 in SO muscle and 86±32 in FG muscle. Despite low GDE activity, the phosphorylase activity was high in chicken muscles. Thus, the calculated PHOS:GDE ratios were very high in chicken muscles: 184±138 in SO muscle and 1289±544 in FG muscle compared to the equivalent in red meat species.

For chickens the samples for phosphorylase activity measurements were selected on the basis of GDE activity (low group and high group). However, the differences in the phosphorylase activity between the resulting groups were quite small (Study III) and thus will not be discussed here any further. The mean values for the PHOS:GDE ratios for chicken muscles presented above are for the low and high group within a muscle. The calculated ratios for low and for high group are given separately in the original paper (Study III).

5.2.2 GDE activity in relation to ultimate pH and glycogen content

There were no significant correlations between the GDE activity and glycogen content, glycolytic potential or pH_u in the different porcine or bovine muscles. When the results for the porcine muscles and also those for the bovine muscles were combined, an inverse trend between GDE activity and pH_u was obtained. The pH_u remained high in the SO muscles where the GDE activity was low. However, there were also differences in the phosphorylase activity and the glycogen content between the individual muscles of a given animal. The GDE activity was similar in *M. longissimus dorsi* of Hampshire cross breed pigs carrying the RN^- mutation (0.232±0.068 Δabs/Δmin) and in the wild type animals (0.230±0.029 Δabs/Δmin) 0.5 hour *post mortem* despite the former having a 77% higher total glycogen content (Study

IV). The high glycogen content in RN^- carriers was a result of an accumulation of both pro-glycogen and macro-glycogen in the muscle.

On the other hand GDE activity in the *M. quadriceps femoris* of chicken was significantly negatively correlated with the glycolytic potential (r = -0.589, P=0.002) and significantly positively correlated with the pH_{25} value (r = 0.478, P=0.018) and pH_u (r = 0.603, P=0.002). A positive correlation was also found between the GDE activity and pH_u (r = 0.416, P=0.048) in *M. pectoralis superficialis*. Furthermore, the GDE activity explained 36% and 38% of the respective variation for pH_u in *M. quadriceps femoris* and in *M. pectoralis superficialis*, respectively. The GDE activity together with GP explained 40% and 61% of the variation in pH_u in *M. quadriceps femoris* and in *M. pectoralis superficialis*.

5.3 The stability of the post mortem GDE activity in pigs with different RN genotypes

The GDE activity was followed from 0.5 h to 48 h *post mortem* in RN^- carrier pigs and in wild type animals (Study IV). The GDE activity was similar in *M. longissimus dorsi* of both genotypes up to 5 hours *post mortem*. Moreover, the transcription levels of GDE for both genotypes were similar in *M. longissimus dorsi* 0.5 h *post mortem*. The GDE activity in *M. longissimus dorsi* of both genotypes decreased slowly as a function of time *post mortem*, and the decrease became statistically significant (P=0.0005 in RN^- and P<0.0001 in wild type) between 5 hours and 24 hours *post mortem*. Furthermore, on the day after slaughter, the GDE activity was significantly (P<0.001) higher in RN^- carriers compared to wild type pigs and this difference was maintained up to 48 h *post mortem*. In both genotypes, only pro-glycogen was degraded after slaughter (from 0.5 to 216 h *post mortem*).

There was a tendency to a decrease in GDE activity with increasing time *post mortem* for the *M. semimembranosus*. However, this difference was significant (p=0.0207) only between 3 h and 48 h *post mortem* in the wild type animals. The GDE activity was similar in *M. semimembranosus* of RN^- carriers 0.170±0.014 Δabs/Δmin and in wild type animals 0.134±0.021 Δabs/Δmin 0.5 h *post mortem*, and not even later the difference between the genotypes was significant. Although the Hampshire cross-breed pigs were raised and slaughtered in Sweden, the values for the GDE activities were very similar to those obtained from pigs in Finland (Table 1).

6 DISCUSSION

6.1 The GDE assay

In the present study, the combined GDE activity (glucosidase + transferase) was measured by a method based on monitoring the shift in the iodine complex spectrum of glycogen limit dextrin. The GDE elongates the outer chains of limit dextrin resulting in the formation of a more 'glycogen like' structure. When limit dextrin is used as a substrate, the iodine complex method is a suitable method for assaying GDE activity for tissue extracts, and also for purified enzyme preparations. The two enzyme activities of GDE act in concert with each contributing to the spectral change produced (Nelson et al., 1970). Therefore the complex iodine method that was used in this study measures the combined GDE activity. For this reason, the GDE activity referred to throughout this work is related to the rate-limiting activity (glucosidase or transferase) in the debranching of glycogen. The combined action of GDE is needed for the break down of the branching point between the glucose chains. It is only when this occurs that phosphorylase is able to continue its action (Brown & Illingworth-Brown, 1966; Hers et al., 1967; Taylor & Whelan, 1968; Nelson et al., 1969). Thus, from the meat science point of view, it is more meaningful to measure the combined activity of GDE rather than its individual activities. The glycogen limit dextrin is a specific substrate for GDE and the other enzymes present in the muscles do not break it down (Nelson et al., 1970). Furthermore, the enzyme has a higher affinity for saccharides with a chain length of four glucose units or greater than for shorter chain lengths (Liu et al., 1995). It is for this reason that the use of artificial substrates for the enzyme may result in an underestimation of the GDE activity.

Various buffers are known to affect the pH optimum of GDE (Gordon et al., 1972). In the present study, the GDE activity was determined with a maleate buffer. Nelson et al. (1969) reported that the optimum pH for GDE activity with a maleate buffer is 6.0 to 6.5 and the activity rapidly decreased below pH 6.0. However, this was not observed in the present study. In the present study, GDE activity was measured by using raw meat extracts as enzyme sources and with the glycogen limit dextrin as the substrate. Raw meat extracts provide a natural environment for this enzymatic action. Consequently, the conditions in raw meat extracts are more similar to those actually occurring in the myofibres than when purified enzyme is used. A situation in living muscle in which all glycogen actually exists as limit dextrin may never occur. Even so in the present study it was focused on the role of GDE in glycogenolysis.

6.2 The effect of pH on GDE activity

The present study showed that the GDE activity is unaffected by pH over the range of 5.0 to 7.4. A short stress just before slaughter reduces the pH in muscle (Tarrant et al., 1972; Henckel et al., 2000) and usually the pH at the time of slaughter is lower than when the

animal is at rest. After slaughter, the pH decrease continues to the pH_u values to around 5.4 (Bendall & Swatland, 1988; Monin, 2004), though the actual pH_u value depends on the muscle concerned. However, a pH decrease of this magnitude was found to have only a minor effect on the GDE activity (Study I).

The shape of the pH-activity profile of GDE in the fast twitch glycolytic (FG) muscle was different to that found in the slow twitch oxidative (SO) porcine muscle. In the porcine FG (*longissimus dorsi*) muscle the maximum GDE activity was determined at about pH 6.5. In the SO (*masseter*) muscle, a relative maximum activity for GDE was not found in the pH range studied and the GDE activity was slightly higher over lower pH values. A wide range for optima pH values within a muscle and the different pH-activity profiles for GDE in *M. longissimus dorsi* and *M. masseter* may suggest that there are different GDE isoforms or different activation mechanisms in these muscles. Taylor and Whelan (1968) suggested that rabbit muscle may contain two GDE isoforms, or be one enzyme with two pH optima. Furthermore, they speculated that in the case of two GDE isoforms, one isoform may have a preference for high-molecular-weight substrates compared to the other. However, during the present study, attempts to influence the pH-activity curve by changing the maleate buffer to phosphate buffer (according to Taylor and Whelan, 1968) or by adding 1 M urea to the reaction mixture (according to Nelson & Watts, 1974) failed to obtain any changes in this curve. Nevertheless, this does not exclude the possibility of the existence of several GDE isoforms in muscles. The human genome contains only one GDE gene (Bao et al., 1996), but there are six different GDE mRNA variations present in muscles (Bao et al., 1997). However, it is not yet known whether all of these mRNA forms are precursors to functional GDE isoform proteins *per se*.

Even though the enzyme activities were measured in samples taken *post mortem*, the results can, to some extent be generalized to give a description that partly includes the living muscles. All mechanisms present in the meat, including enzyme activities, are optimal for satisfying the needs of a living muscle fibre and therefore do reflect the situation in a living muscle. In the present study, the physiological aspects were taken into consideration in order to give a wider perspective in order to interpret the results obtained.

During intensive exercise, the pH of living muscle may decrease from above 7 (pH of resting muscle) to values 6.5 or 6.3 in fatigued muscle (Lovell et al., 1987; Juel, 1996). Hence, GDE must have a capability for being active over a wide range of pH values to ensure continuous glycogenolysis and glycolysis in order to maintain the energy production to support muscle contraction. The present study showed that it has this capability. Furthermore, an increase in GDE activity with decreasing pH in SO muscle could reflect the capability to maintain the enzyme activity during intensive muscle exercise. In the living animal the lactate and protons formed in type IIB muscle fibres are transported out of the fibres, and into either the bloodstream or into type I muscle fibres (Halestrap & Price, 1999), resulting in a pH decrease in type I fibres. Type I muscle fibres are able to produce energy from lactate by oxidation, thus the predominantly SO muscles do not need to use glycogen as an energy source when the pH is high (Briskey, 1964), i.e. the GDE activity is not critical. However, SO muscles must have the capability to break down glycogen in conditions of low pH, if their oxygen stores are depleted. The low pH maximum for GDE activity found in the present

study enables the glycolysis in low pH values. During continuous exercise, a situation may arise in which the type IIB fibres become exhausted, but the type I fibres must continue to function. In such situations energy can still be produced anaerobically from glycogen, and thus the activity of glycogenolytic enzymes at low pH-values is essential.

6.3 The effect of temperature on GDE activity

6.3.1 GDE activity at temperatures near to body temperature

The GDE was most active in the *M. longissimus dorsi* at temperatures around 40 °C, whereas the optimum temperature for the *M. masseter* GDE was higher, between 40 and 50 °C. In both muscles, a decrease in temperature to below the normal pig body temperature (which is 38.5±0.7 °C according to Hannon, Bossone & Wade, 1990) induced a rapid fall in GDE activity. Moreover, phosphorylase is most active at normal body temperatures (Cori et al., 1943). The decrease in GDE activity was significant when the temperature decreased from 39 °C to 25 °C in *M. masseter* ($P=0.0015$) or from 39 °C to 15 °C in *M. longissimus dorsi* ($P<0.0001$). In both muscles, the GDE was virtually inactive at temperatures below 15° C.

A change in muscle temperature of just a few degrees has a significant effect on the rate of *post mortem* glycolysis. Klont and Lambooy (1995) reported that an increase in pre-slaughter muscle temperature from 37 to 39 °C accelerated the *post mortem* metabolite breakdown and pH decrease in halothane positive and halothane gene carrier pigs. A temperature rise to above 40 °C in muscle strips also accelerated the *post mortem* metabolism of halothane negative pigs (Klont et al., 1994). The present study showed that GDE activity was quite stable when temperature was varied a few degrees around normal body temperature. Thus, the faster rate of glycolysis in high temperatures is not a consequence of a change in GDE activity, however, GDE does either prevent it. In fast glycolysing Poland China pigs the *post mortem* muscle content of free glucose and G-1-P rose faster than in slow glycolysing Hampshire or Chester White pigs (Kastenschmidt et al., 1968). This indicates both higher GDE and higher phosphorylase activities in relation to phosphofructokinase activity in fast glycolysing animals than in slow glycolysing animals. It is probably the substrate availability rather than the temperature rise of few degrees that controls the GDE activity when muscle temperature is close to body temperature.

Exercise, pre-slaughter stress and fast rate of carbohydrate catabolism increase muscle temperature *pre mortem* as well as *post mortem* (Lovell et al., 1987; Offer, 1991; Bowker et al., 1999; Lindahl et al., 2006). Muscle temperature may rise after slaughter as much as 3 °C in fast glycolysing pigs, particularly when the chilling is delayed (Bendall, 1973; Offer, 1991). The present study showed that an increase in temperature of this magnitude does not inhibit GDE activity. On the contrary, GDE activity may slightly increase with a rise in temperature of few degrees to above normal body temperature.

6.3.2 The effect of temperature decrease on GDE activity

Contrary to the lack of an effect of pH on GDE activity, temperature decrease has a great effect on the GDE activity in porcine muscles. It is well documented that the rate of glycolysis decreases with time *post mortem* (Bendall, 1973; Jolley et al., 1981; Schäfer et al., 2002; Young et al., 2002) mainly as a result of muscle temperature decrease during chilling. Like all chemical reactions, *post mortem* glycolysis and the resultant onset of *rigor mortis*, are temperature dependent. Therefore they are relatively fast at normal body temperatures and slower as the temperature decreases to about 10 °C (Bendall, 1951; Bendall, 1973). However, the rate of glycolysis increases again when the temperature is further lowered to around 0 °C.

A rapid decrease in temperature to around zero degrees when there is still ATP left induces an inhibition on the action of calcium pumps following an increase in Ca^{2+} content in the fibre (Bendall, 1975). This is especially the case in slow twitch oxidative muscles. Due to excess Ca^{2+} in the fibres, the initial rate of glycolysis at around zero degrees in rapidly chilled meat is similar or even higher than at 37 °C (Bendall, 1973; Jolley et al., 1981). However, this increased rate of glycolysis is maintained for only a short period (the first 3-4 hours *post mortem*) and thus in isolated *pre rigor* bovine muscle, it takes about 6 h at 30 °C to reach pH 5.9, whereas at 0 °C the same pH is not reached until 24 h (Honikel, 1992). This might indicate that the increased phosphorylase activity is responsible for the increased rate of initial glycolysis at low temperatures, whereas the curtailing of GDE activity restrains glycolysis at a later phase. The action of phosphorylase alone is able to break down a maximum of 34.6% of muscle glycogen (Meléndez-Hevia et al., 1993). Thus, albeit the muscle glycogen content at the time of slaughter is normal, and muscle buffering capacity is 47 to 52 mmol H^+/(pH*kg meat) (Kivikari, 1996; Kylä-Puhju et al., 2004) the glucose available for the action of phosphorylase is unable to decrease the pH lower than 6. After that GDE activity is needed to continue glycolysis. However, the present study showed that the GDE activity is very low at temperatures below 15 °C and therefore such temperatures may considerably delay glycolysis when reached during chilling.

The present study showed that the GDE activity started to decrease when the temperature was lowered to below 35 °C. In rabbit muscle, the temperature optimum for GDE activity is near 50 °C, and the activity decreases sharply when the temperature decreases to 20 °C (Nelson & Watts, 1974), which is consistent with the results for the porcine *M. masseter* of the present study. It is very likely that carcass chilling causes a decrease in GDE activity after slaughter. Thus, the GDE activity in the carcass may not be maximal when it is needed to continue glycogenolysis. This may lead to a delay in the rate of glycogenolysis and glycolysis *post mortem* depending on: pre-slaughter glycogen content, rate of *post mortem* glycolysis and also the chilling rate. However, in many cases pH continues to fall even when the temperature in muscles has dropped below 15 °C. The GDE has an effect on glycolysis only in situations where the phosphorylase has revealed the branching points of glycogen. Thus, the glycolysis and pH decrease in temperatures below 15 °C may be enabled by phosphorylase action alone when the outer glucose chains of glycogen are long enough. However, if the branching points of glycogen are attained, the low GDE activity in low temperatures may stop the glycolysis.

6.3.3 The concurrent effects of pH and temperature on GDE activity

The rate of pH decrease and the rate of temperature decrease combined affect meat quality. Low pH combined with high muscle temperature during the early *post mortem* period may produce PSE meat (Bendall & Wismer-Pedersen, 1962; Bendall & Swatland, 1988; Offer & Knight, 1988b; Offer, 1991; Joo et al., 1999; van Laack et al., 2000; Sams & Alvarado, 2004). Chilling rate influences pork quality, as it depends on the pH and temperature histories of the muscle (Bendall & Swatland, 1988). In addition in some cases PSE could be avoided by rapid chilling (Honikel, 1987; Offer & Knight, 1988b; Offer, 1991; Schäfer et al., 2002; Sams & Alvarado, 2004). The present study showed that the pH decrease alone had only a minor effect on GDE activity, whereas the temperature decrease significantly lowered the activity. Thus, it is the chilling rate rather than the muscle pH decrease that defines the *post mortem* GDE activity. One explanation for the positive effects of rapid chilling on meat quality could be the inactivation of GDE leading to a restraining or slowing of glycolysis in porcine muscles. Thus the slowing of GDE gives time for the temperature to decrease before pH reaches the critical value of 5.8. Adequate chilling determines the rate of *post mortem* glycolysis and allows the development of meat at optimum quality. However, if muscle temperature drops below 7 °C when pH is higher than 5.7 (Hannula & Puolanne, 2004) or 6.0 (Pearson & Young, 1989a) and there is still ATP available for muscle contraction, there will be a risk of cold shortening and increase in toughness of meat.

The GDE may also play a role in the phenomena of so called ‘heat ring’, which is known to occur in fast chilled beef, particularly in the quicker cooled outer parts of *M. longissimus dorsi*. Rapid cooling leads to a reduction in the rate of pH fall in the outer parts of *M. longissimus dorsi* and causes formation of a darker band in muscle and consequently an unattractive appearance (Orcutt et al., 1984; Warriss, 2000). The reduction in the rate of glycolysis could be a result of decreased GDE activity due to low temperature. It should be pointed out that the rates of pH and temperature decreases vary widely within a muscle during cooling (Puolanne & Ruusunen, 1998). These values can also vary widely within carcasses and between carcasses. Therefore all these considerations make the monitoring of these aspects difficult under practical working conditions.

In summary, the GDE is most active when the temperature is around normal body temperature. The pH decrease in the range found in meat does not significantly influence on GDE activity. Thus the GDE activity probably does not prevent rapid glycogen degradation at high temperatures, in conditions which may lead to formation of PSE meat. However, the GDE activity may control the rate of glycogenolysis during late phase of cooling, and in very fast chilled meat it may even stop the glycolysis completely. In other words, the improved meat quality accomplished by rapid cooling may be due to a restraining of the glycolysis by inhibition of GDE activity. The actual effect of GDE on glycolysis depends, however, the amount of glucose units in glycogen which are directly available for phosphorylase.

6.4 The activity of glycogenolytic enzymes in the muscles of meat animals

After slaughter, muscle ATP level is mainly maintained at a constant level by glycolysis which is fuelled by the anaerobic degradation of glycogen (Bendall, 1973; Hamm, 1977). Thus, the rate of *post mortem* glycolysis is determined by the rate of the hydrolysis of ATP (Bendall, 1973; Scopes, 1974; Hamm, 1977). Moreover, the muscle aerobic capacity (Livingston & Brown, 1981; Pearson & Young, 1989b; Hamm & El-Badawi, 1991) and muscle PCr content at the moment of slaughtering (Bendall, 1951; Lawrie, 1953; Bendall, 1973; Hamm, 1977; Bertram et al., 2002; Henckel et al., 2002; Pösö & Puolanne, 2005) influence the rate of pH decrease, rather than the content of glycolytic enzymes *per se* (Scopes, 1974; Allison et al., 2003). On the other hand, substrate availability has an impact on the rate of glycolysis (Warriss et al., 1988; Henckel et al., 2000; Daly et al., 2002; Henckel et al., 2002; Berri et al., 2004) indicating that glycolytic or glycogenolytic enzyme activity may still have a role in the rate of decrease in pH.

The differences in glycogen catabolism in muscles of three meat producing species were investigated (Studies II and III). The GDE from FG porcine and bovine muscles rapidly breaks down its target substrate, glycogen limit dextrin. However, GDE activity was about twice as high in porcine muscles as in their corresponding bovine muscles (Study II). The GDE activity in chicken SO *M. quadriceps femoris* (Study III) was close to the values obtained in porcine and bovine SO muscles, but in *M. pectoralis superficialis* the activity was very low compared to porcine or bovine FG muscles. In pigs and in cattle, the GDE activity was twice as high in the FG muscles as in the SO muscles. This is consistent with the study of Tsutou et al. (1985), who obtained similar results with rabbit slow and fast muscles. In contrast, the GDE activity was lower in the very glycolytic *M. pectoralis superficialis* in chicken (Study III), than in the more oxidative *M. quadriceps femoris*, a finding which was exactly the opposite to the equivalent findings in pigs and cattle.

Despite the low GDE activity, the phosphorylase (*a*+*b*) activity was high in chicken muscles indicating high glycolytic activity. This is consistent with the rate of *post mortem* glycolysis being much faster in poultry muscles than in red meat species (Addis, 1986). In the present study, only selected samples were analysed for phosphorylase activity (five low GDE activity and five high GDE activity samples) in chicken. However, no relationship between the phosphorylase and GDE activities was found in this species. Hence, the selection of chicken phosphorylase samples is not discussed here any further. Compared to chicken muscles, the phosphorylase (*a*+*b*) activity was intermediate in porcine muscles and low in bovine muscles. This is consistent with the results from previous studies, which reported that the activities of glycolytic enzymes, such as phosphorylase, glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase are higher in porcine muscles than in bovine muscles depending, however, on the muscle concerned (Laborde et al., 1985; Talmant et al., 1986; Hamm & El-Badawi, 1991). The result about the differences in phosphorylase activity between species is parallel with the generally observed order of the rate of *post mortem* pH decline in the main meat species: poultry>pork>lamb>beef (Pearson & Young, 1989a; Monin, 2004) (Lundberg et al., 1987; Warriss, 2000).

The phosphorylase (*a*+*b*) activity was lower in SO muscles than in FG muscles of pig, cattle and chicken (Studies II and III) indicating lower glycolytic activity. The result is in accordance with the results of Monin et al., (1987) who found that phosphorylase activity (*a*+*b*) decreases with the increase in the SO character of pig muscles. The generally lower activities of enzymes in the glycogen to lactate pathway in SO muscles compared to that in FG muscles is well documented (Beecher et al., 1965b; Laborde et al., 1985; Talmant et al., 1986; Monin et al., 1987; Pearson & Young, 1989b; Hamm & El-Badawi, 1991), as is the high capacity for aerobic oxidation of glucose and fats in SO muscles. The pre-slaughter handling and treatment of animals may not influence the activity of glycogenolytic enzymes. A reduction in pig muscle glycogen stores by strategic feeding did not result in adaptive changes in phosphorylase or phosphofructokinase (Rosenvold et al., 2001). Furthermore, Fernandez et al. (2002) reported that pre-slaughter handling did not affect pig muscle LDH, CS or phosphorylase (*a*+*b*) activities. All animals in the present study were slaughtered in commercial slaughterhouses.

In agreement with several previous studies (Talmant et al., 1986; Monin et al., 1987; Warriss et al., 1988; Lefaucheur et al., 1991; Sams & Janky, 1991; Karlsson et al., 1993; Przybylski et al., 1994; Wittmann et al., 1994; Karlsson et al., 1999; Fischer & Dobrowolski, 2002) the glycolytic potential (GP) was lower in SO than in FG muscles of pig, cattle and chicken. Rapid glycolysis is not essential for SO muscles in living animals as these muscles normally utilize energy for muscle contraction aerobically. Compared to FG muscles, SO muscles have a better blood supply, higher myoglobin content, more mitochondria and a more active catabolic pathway of glycogen to CO_2 and H_2O in Krebs cycle (Beecher et al., 1965a; Beecher et al., 1965b; Bendall, 1975; Laborde et al., 1985; Talmant et al., 1986; Pearson & Young, 1989b; Ruusunen, 1994; Ruusunen & Puolanne, 2004). The fast glycolysis in FG muscles is enabled by a high GP in association with the high activities of glycogen degrading enzymes, allowing a rapid conversion of glycogen into lactate. The present study showed that besides the phosphorylase activity, GDE activity was also higher in the FG muscles than in the SO muscles of pig and cattle. Although in chicken FG *M. pectoralis superficialis* the GDE activity was low, the phosphorylase (*a*+*b*) activity was high enabling rapid glycolysis.

In summary, in pig or cattle GDE and phosphorylase activities increased with the fast twitch and glycolytic properties of a muscle of a given animal. In chicken, the phosphorylase activity also increased but the GDE activity decreased with the FG properties of a muscle. GDE activity was about twice as high in porcine muscles than in the corresponding bovine muscles. In the porcine muscles, the phosphorylase activity was also somewhat higher than in bovine muscles indicating higher capacity to degrade glycogen. In chicken muscles, GDE activity was low but the phosphorylase (*a*+*b*) activity was high compared to either porcine or bovine muscles. The high phosphorylase activity enabled a fast glycolysis in chicken muscles.

6.4.1 GDE activity in relation to the phosphorylase activity

There was an increase in both GDE and in phosphorylase (*a*+*b*) activities with the fast twitch and glycolytic character of muscles in pigs and cattle. Even so, the increase in the phosphorylase activity was more pronounced than that of GDE. In chicken muscles the GDE activity was low, but the phosphorylase activity was high, especially in *M. pectoralis superficialis*. The ratio (PHOS:GDE) between the two enzymes involved in glycogen breakdown was calculated for every muscle studied (Studies II, and III). This was to compare the relative capacity of the muscles to catabolize glycogen. The GDE and phosphorylase activities were not measured in the same units, thus the ratios are only useful to compare the relative differences in activity between these enzymes between muscles not as the true difference between the activities of these enzymes within a muscle.

In all species the PHOS:GDE ratios were found to be higher in the FG muscles than in the SO muscles. The ratio was lowest in the bovine SO *M. masseter* and highest in the chicken FG *M. pectoralis superficialis*. The values of the ratios in chicken muscles were multiple to those in the muscles of pigs and cattle. In porcine muscles the ratio was relatively constant within a muscle type.

FG muscles are capable of a short-term strenuous contractile activity, but they fatigue quite easily (Lawrie, 1985). During strenuous physical activity, a high PHOS:GDE ratio in FG muscles should enable a short burst of glycolysis, which leads to a rapid increase in H^+ production. Skeletal muscles have a great excess in capacity for glycolytic energy production. Saltin and Gollnick (1983) calculated that a full activation of phosphofructokinase and phosphorylase, which are generally considered to be regulators in the flow of substrate through the glycolytic pathway, would result in massive lactate production, 50 and 100 mmol lactate/kg/min in human SO and FG fibres respectively. Furthermore, it has been estimated that a 5 % activation of phosphorylase could account for the maximum lactate production in skeletal muscle if the rate of glycolysis were depended on phosphorylase (Saltin & Gollnick, 1983). The advantage of excess capacity is that with high enzyme levels, it is possible to attain high rates of substrate fluxes also at low substrate contents. In the living fibres, intracellular pH and inorganic ion contents are kept within narrow ranges because of a need to maintain critical conditions for optimum macromolecular function including that of proteins (Somero, 1986). Since phosphorylase initiates the release of considerable amounts of chemical energy, there must be safeguards to prevent its uncontrolled activity and to control the consequences of fast glycolysis. The high buffering capacity of FG muscles (Davey, 1960; Talmant et al., 1986; Rao & Gault, 1989; Puolanne & Kivikari, 2000; Kylä-Puhju et al., 2004) protects these muscles against a sudden pH decrease, but the relatively low GDE activity compared to the phosphorylase activity may be needed as a further restraint to glycogenolysis. Based on the finding of a high PHOS:GDE ratio in FG muscles, it seems that the GDE activity restricts the rate of glycolysis in FG muscles more than it does in SO muscles.

In chicken muscles, there was an even greater discrepancy between the GDE and phosphorylase (*a*+*b*) activities, in that there was a much higher PHOS:GDE ratio than in porcine or bovine muscles. This may be explained by the normal function of muscles in the live chicken. Muscles with a high proportion of FG fibres, such as chicken *M. pectoralis*

major, are known to contract rapidly in short bursts and are relatively easily fatigued (Barbut, 2002). These kinds of muscles rely on glycogen and anaerobic glycolysis for additional energy production during stressful situations. Chicken *pectoralis* muscle is incapable of sustaining prolonged flight; chickens tend to run rather than fly to escape danger and are able to fly only short distances at a time (Baynes & Dominiczak, 1999). The low GDE activity of chicken muscles found in the present study seems to indicate that the amount of glycogen directly available for phosphorylase is sufficient for short flights, and the GDE may be unnecessary in the 'fight-or-flight' reaction. The unavoidable resting period after a flight may be due to the restriction of glycogenolysis by low GDE activity and be essential for transporting accumulated lactic acid away from the muscle fibres, thus minimizing fibre damage. Nevertheless, the presence of some GDE activity in the muscles may indicate that the chicken is capable of additional, but lighter exercise after a short rest. Further, the higher PHOS:GDE ratio in *M. pectoralis superficialis* relative to that of the *M. quadriceps femoris* may indicate that GDE activity limits the rate of glycogenolysis more in *M. pectoralis superficialis*. Although the buffering capacity of chicken *pectoralis* muscle is high compared to the more aerobic chicken muscles or to mammalian muscles (Kivikari, 1996), it may not be sufficient to protect the muscle from fast pH changes. The restriction of the rate of glycolysis by low GDE activity may therefore be essential to protect the muscle from devastation.

The present study suggests that a high buffering capacity together with low GDE activity may be an important protection mechanism of a living muscle against an excessively fast pH decrease. Furthermore, the GDE might restrict the rate of glycogen degradation more in FG muscles than in SO muscles.

6.4.2 GDE activity in relation to ultimate pH and glycogen content

The pH_u was lower in FG muscles than in SO muscles of pig, cattle and chicken (Studies II and III). This agrees with numerous earlier studies (Beecher et al., 1965b; Hunt & Hedrick, 1977; Laborde et al., 1985; Talmant et al., 1986; Monin et al., 1987; Bendall & Swatland, 1988; Warriss et al., 1988; Jones & Grey, 1989; Rao & Gault, 1989; Fernandez & Tornberg, 1991). Furthermore, several studies have reported that high *post mortem* temperatures and slow chilling accelerated glycolysis and pH decreases and hence fewer hours were needed to achieve pH_u whereas low temperatures retard the rate of glycolysis (Bendall, 1951; Beecher et al., 1965a; Bendall, 1973; Renou et al., 1986; Pearson & Young, 1989a; Jones et al., 1993; Josell et al., 2003b). The effect of temperature decrease may be different in different types of muscles. The present study (I) showed that a temperature decrease to below 25 °C in SO or below 15 °C in FG porcine muscle inactivates the GDE. The temperature decrease induced a faster decrease in the GDE activity to below determination limit and probably a stronger decrease in the rate of glycogenolysis in the SO muscles than in FG muscles. In agreement with that, Sams and Janky (1991) found that chilling slowed down the *rigor mortis* development in broilers' SO muscles but not in their FG muscles. On the other hand, another study showed that, the *post mortem* pH decrease at 25 °C was faster in rabbit FG muscle than in SO muscle, but the difference disappeared when temperature decreased to 15 °C (Renou et al., 1986).

The GDE activity may play a role in controlling the extent of *post mortem* pH decrease. Compared to *M. longissimus dorsi*, *M. masseter* cools down faster due to its size and location. It also has a slower pH decrease and higher pH_u. Even though the muscle glycogen content *per se* is not a limiting factor (Study II), the reported pH decreases in SO muscles usually ceases around 6.0: range 5.9 to 6.1 in the *M. infraspinatus* (Warner et al., 1993; Kylä-Puhju et al., 2004), 5.9 in the *M. masseter* (Kylä-Puhju et al., 2004), over the range of 5.9 to 6.2 in the *M. semispinalis* (Lefaucheur et al., 1991; Warner et al., 1993; Przybylski et al., 1994), and 6.2 in the *M. vastus intermedius* (Bendall, 1979). One reason for the high pH_u in the *M. masseter* could be the rapid temperature decrease during cooling, which lowers GDE activity in the muscle and thus may delay glycogenolysis. This is consistent with the calculations (Study I) that the amount of glycogen directly susceptible for the degradation of phosphorylase in the *M. masseter* is large enough to cause a *post mortem* pH decrease from 7 to about 6. After that the GDE activity is needed so that glycogenolysis can continue. Furthermore, Study I showed that a decrease of about 15 degrees in temperature in *M. masseter* from the level prevailing in a living muscle practically inactivates the GDE whereas in the *M. longissimus dorsi* it takes a decrease of over 20 degrees to obtain a similar effect. The above is consistent with the results of Beecher et al. (1965a) who reported that the pH_u of the dark part of the porcine *M. semitendinosus* was significantly lower in muscles kept at 37 °C than in other samples kept at 4 °C. However, when the light portions of *M. semitendinosus* were kept at 37 °C or 4 °C, no significant differences between the pH_u values were found.

The results of the present study indicated that pH_u may be affected in rapidly chilled muscles due to inhibition of GDE activity. The effect of temperature decrease on GDE activity was more pronounced in the SO muscles than in the FG muscles. Unfortunately recent studies relating to the carcass chilling rate have mainly focused on FG muscles. Jones et al. (1993) reported that accelerated chilling led to slower pH fall and higher pH_u in porcine *M. longissimus dorsi* and *M. semimembranosus*, whereas Springer et al. (2003) found similar pH_u values in corresponding muscles despite differences in chilling speed. Pösö and Puolanne (2005) carried out a meta-analysis of the relatively extensive literature on very fast chilled beef, which revealed that in many cases, very fast chilling gives rise to beef with pH_u ca. 0.1 to 0.3 units higher than conventional chilling. This could be a result of an inhibition of the GDE activity due to the temperature decrease. However, the pH_u was not affected by the chilling rate in all studies in the meta-analysis, as in some studies the effect was even the opposite. Sometimes there was only an initial reduction in pH fall suggesting that the remaining phosphorylase and GDE activity after exposure to low temperature might be sufficient if the available time was long enough. The evaluation of GDE activity and its importance in the attainment of pH_u is not simple. The length of the A-chains of glycogen (the amount of glucose directly susceptible for phosphorylase action) at death and the time interval for muscle temperature to reach 15 °C in FG muscles or 25 °C in SO muscles have great relevance in determining the possible role of GDE activity in the extent of pH decrease.

The GDE activity did not correlate with the GP, the pH_{35} or with the pH_u value for either porcine or bovine muscles (Study II). Nevertheless, a negative trend between the GDE activity and pH_u was obtained when the results for individual muscles of a given species were combined. The pH_u was high in SO muscles where the GDE activity was low compared to FG

muscles. However, it should be borne in mind that there were also differences in glycogen content and phosphorylase activity between the different muscles of a given animal.

In chicken, GDE activity correlated positively with pH_u in both muscles. The GDE alone explained 36% of the variation in pH_u in the SO muscle of chicken and 38% in their FG muscles, while GDE activity together with glycolytic potential accounted for 40% and 61% of the variation in pH_u in the respective muscles. However, the high pH_u and low GP in chicken muscles in the present study may limit the extent of these generalizations. Furthermore, the positive correlation between GDE and pH_u could be a result of intense *pre mortem* glycolysis, which would have led to a lower glycogen content at slaughter in fast glycolysing animals than in slow glycolysing animals of the same species. Thus, it is possible that the correlation between GDE activity and pH_u is the opposite in animals slaughtered under minimal stress. In line with the assumption of animals being stressed before slaughter, the correlation between GDE activity and GP was negative in chicken SO muscle. Chickens and pigs are more sensitive to pre-slaughter stress than cattle. Their muscles are very glycolytic and glycogen reservoirs begin to degrade even under light stress, whereas in the more oxidative bovine muscles the breakdown of glycogen begins as a consequence of long-term stress (Lawrie, 1985; Henckel, 2002). The GP of bovine FG *M. longissimus dorsi* was higher than in the corresponding porcine muscle or in the chicken *M. pectoralis superficialis*.

In general, pH_u is higher in chicken muscles than in cattle or pig (Monin, 2004). The reduced extent of decrease in pH in chickens is a result of lower glycogen content, higher buffering capacity and higher initial pH than in red meat species (Kivikari, 1996; Papinaho et al., 1996). In the present study, the pH_u values of chicken, especially those in SO muscle, were even higher than those usually reported in the literature. According to Jones and Grey (1989), the pH_u in chicken leg meat ranges from 6.1 to 6.4. In the present study, 25 min after slaughter the SO leg muscle had attained its pH_u value of 6.89. This high pH_u value could be a result of exhaustion of the animals and rapid glycolysis before slaughter, which would have diminished glycogen stores in the muscles. In the present study, the GP was low in the chicken SO muscle, and the glycogen content at 25 min *post mortem* was 14.1 mmol/kg muscle, which is close to the minimum residual glycogen content of 10 mmol/kg, reported for bovine muscles (Immonen & Puolanne, 2000). Henckel (2002) observed that a considerable number of chickens were already metabolically exhausted at sticking resulting in high pH_u values. He concluded that chickens are more susceptible to stress in comparison to other meat producing animals, probably because of their normal behaviour pattern. Chicken muscles are also characterised by a very high number of glycolytic fibres. Moreover, the distribution of fibre type, enzyme activity and capillary supply in this species, all exacerbate the effect of stress. In Study III, chickens were fasted 13 to 16 hours before slaughter. According to Warriss et al. (1988), food withdrawal pre-slaughter decreases glycogen content in chicken SO muscles but not in their FG muscles. The fact that SO muscles in the chicken reached pH_u before sampling may have influenced their respective GDE activities. Nevertheless, the GDE activity was higher in SO muscle than in FG muscle. In the chicken FG muscles, the pH_u was 6.03, which is close to the mean value of 5.96 ± 0.04 (SE) that van Laack et al. (2000) reported for normal *pectoralis* muscle and within the range of 5.56 to 6.42 (mean 5.83) reported by Barbut (1997a). However, values close to the lower end have been reported more frequently (Jones & Grey, 1989; Guarnieri et al., 2003).

6.4.3 Effects of pig genotype on GDE and meat quality

A dominant RN^- allele of PRKAG3 gene present in Hampshire and Hampshire cross-breed pigs unfavourably affects meat quality (Fernandez et al., 1992b; Estrade et al., 1993; Sellier & Monin, 1994; Enfält et al., 1997b; Josell et al., 2003a; Josell et al., 2003b; Lindahl et al., 2004). The RN^- carrier pigs have about a 70% higher glycogen content in FG muscles than in the corresponding muscles of wild type animals (rn^+ homozygous pigs) (Estrade et al., 1993; Enfält et al., 1997a; Le Roy et al., 2000; Essén-Gustavsson et al., 2005). Glycogen exists in two forms in skeletal muscle: high molecular weight macro-glycogen and low molecular weight pro-glycogen (Lomako et al., 1991; Lomako et al., 1993). In the present study, glycogen content was 77% higher in RN^- carriers than in wild type animals (Study IV) and the difference was a result of the accumulation of both glycogen types in the muscle. In contrast, Essén-Gustavsson et al. (2005) reported that a high glycogen content in RN^- carriers is exclusively due to increased macro-glycogen content. However, the pigs in that study were younger and lighter than in the present study. In RN^- carriers the proportion of macro-glycogen was higher than in the wild type animals (Study IV), which is consistent with several studies showing that the macro-glycogen fraction increases with increasing muscle glycogen content (Adamo & Graham, 1998; Adamo et al., 1998; Asp et al., 1999; Derave et al., 2000; Shearer et al., 2000). The pro-glycogen to macro-glycogen ratio in wild type (Hampshire x Swedish Landrace x Yorkshire) pigs was similar to that reported for (Danish Landrace x Danish Yorkshire x Duroc) crossbreed pigs by Rosenvold et al. (2003). Furthermore, Study IV showed that irrespective of genotype, only pro-glycogen is used from 0.5 to 48 h (and up to 216 h) *post mortem* for muscle energy production. This is consistent with the findings of Rosenvold et al. (2003) who observed degradation of only pro-glycogen during the first 45 minutes after slaughter in wild type animals.

Despite differences in the glycogen content, shortly after slaughter (0.5 h) GDE activity and the transcription level of GDE were similar in both genotypes. Estrade et al. (1994) also reported uniform GDE activity in RN^- carriers and in wild type animals 5 min *post mortem*. Furthermore, they found similar phosphorylase activity in the different RN genotypes. Nevertheless, the initial decrease in pH was faster in *M. longissimus dorsi* from RN^- carriers than in wild type animals (Study IV). Carbohydrate availability has a great effect on the rate of pH decrease in porcine and chicken muscles (Warriss et al., 1988; Henckel et al., 2000; Henckel et al., 2002; Berri et al., 2004). In pigs slaughtered under minimal stress, the rate of pH decrease during the first 45 minutes *post mortem* was 0.55 units/hour in the *M. longissimus dorsi*, 0.80 in the *M. biceps femoris* and 0.49 in the *M. semimembranosus* (Henckel et al., 2000). Furthermore, these authors reported that when pigs were slaughtered after the glycogen contents of their muscles had lowered by subjecting the animals to adrenaline injection and treadmill exercise well before slaughter, the rate of pH decreased was only 0.37, 0.41 and 0.36 units/hour in the same muscles, respectively. Moreover, Daly et al. (2002) reported that during chilling the bovine *longissimus dorsi* muscle reaches pH 6 in higher temperatures when it contains high glycogen content than those muscles that contain less glycogen. Thus, *post mortem* glycolysis was faster in animals having more glycogen indicating that low substrate availability may slow down glycolytic rate.

The reduced rate of glycogenolysis observed when the glycogen content is low may be due to a rate limiting effect of GDE. Muscles with reduced glycogen stores end up in a situation where glycogen limit dextrin has to be broken down to maintain the ATP level. At this point GDE determines the rate of the pH decrease rather than phosphorylase. Furthermore, Lees et al. (2004) studied rat skeletal muscle sarcoplasmic reticulum and demonstrated that an activity that leads to a reduction in muscle glycogen content also reduces GDE content. The present study was carried out with carcasses randomly selected from the slaughter line and thus representing a normal commercial situation. The GDE activity may neither slow down nor stop the glycogenolysis in normal porcine or bovine muscles during the normal *post mortem* metabolic reaction sequence. However, the possibility that the GDE activity has a critical role in glycogenolysis can not be excluded. The role of the GDE in glycolysis is important when the carcass is chilled rapidly, since in the present study it was showed that a decrease in muscle temperature radically decreases the GDE activity (Study I).

6.5 Stability of the GDE activity *post mortem*

The stability of the GDE activity *post mortem* was studied in the *M. longissimus dorsi* and *M. semimembranosus* of pigs with different RN-genotypes (Study IV). It was found that the glycolytic muscles of RN^- carriers possess a higher glycolytic potential, a faster initial decrease and a lower pH_u than wild type animals (rn^+), which agrees with earlier studies (Fernandez et al., 1992b; Estrade et al., 1993; Enfält et al., 1997b; Le Roy et al., 2000; Josell et al., 2003a; Josell et al., 2003b; Lindahl et al., 2004).

The decrease in pH was faster and the pH_u lower in *M. longissimus dorsi* of RN^- carriers than in wild type animals, which agrees with earlier findings (Enfält et al., 1997b; Josell et al., 2003a; Josell et al., 2003b; Lindahl et al., 2004). However, despite differences in the pH decrease patterns and glycogen contents, it seems that immediately after slaughter the glycogenolytic enzymes are no more active in RN^- carriers than in wild type animals (Estrade et al., 1994). The present study showed that GDE activity was similar in both genotypes and remained at the level found shortly after slaughter for the first five hours *post mortem*. However, the GDE activity was significantly reduced at 24 h *post mortem* in *M. longissimus dorsi* of both genotypes. The decrease was more pronounced in wild type animals. In wild type animals at 24 h *post mortem* the GDE activity had decreased 64% from the initial value, while in the RN^- carriers the decrease was only 40%. This significant ($P<0.01$) difference in GDE activity between genotypes was maintained for up to 48 h *post mortem*. The extended pH decrease in RN^- carriers compared to the wild type could be a result of the prolongation of the high GDE activity in these animals. Furthermore, in RN^- carriers the pH_u is attained not until 48 h *post mortem* (Lindahl et al., 2004). In the present study, however, also the wild type animals (*M. longissimus dorsi*) needed 48 h to attain pH_u. No significant differences in the *post mortem* GDE activity (from 0.5 h to 48 h) of *M. semimembranosus* were found between the two genotypes. There was a tendency towards a decrease in GDE activity in the *M. semimembranosus* with time *post mortem*. However, this decrease in GDE activity only became significant in the muscles of the wild type animals at 48 h *post mortem*. The pH

decrease pattern and pH_u were similar in *M. semimembranosus* from RN^- carriers and from wild type animals.

The differences between *M. longissimus dorsi* and *M. semimembranosus* offer indirect support for the importance of GDE activity in the development of pH_u between the RN genotypes. The change in GDE activity was followed from 0.5 h to 48 h *post mortem*. The GDE activities were stable, particularly in the *M. semimembranosus* of RN^- carriers, where no significant decrease in activity with time was found. Furthermore, the GDE activity, the rate of pH decrease or pH_u in *M. semimembranosus* did not differ between the RN genotypes, whereas these values did differ for the *M. longissimus dorsi* muscle. It therefore seems that GDE activity might be related to the extent of pH decrease, and it remains to be investigated if a difference in the GDE activity late *post mortem* between the RN genotypes would also lead to differences in the pH_u of the *M. semimembranosus*. Lindahl et al. (2006) who also participated in some of the same work that this study cover, compared pH decreases between the RN^- carriers and wild type animals, but with a larger number of pigs. They found a faster pH decline and lower pH values at 3 and 5 h *post mortem* in the *M. semimembranosus* of the RN^- carriers than in wild type animals. However, they found no difference in the pH_u. Despite similarities between the GPs, the activity of glycogenolytic enzymes (Fischer & Dobrowolski, 2002; Study II) and fibre type composition (Ruusunen & Puolanne, 2004) between the porcine *M. longissimus dorsi* and the *M. semimembranosus*, the pH decrease was found to be slower in the *M. semimembranosus* (Henckel et al., 2000). However, considering the data obtained from the present study on these muscles, the results support the conclusion that GDE activity does affect the pH decrease *post mortem*. As shown in Study I the pH itself has only a minor effect on the GDE activity when its range lies between 5.5 and 7.

The sustained high GDE activity especially that found in RN^- carriers, may result from high muscle glycogen content, since it has been shown that binding to glycogen limit dextrin, or to a lesser extent, to glycogen alone, protects the GDE from denaturing agents (Gillard et al., 1980; Scraba et al., 1988). The sustaining of high GDE activity in muscles for a lengthy period after slaughter, as such, is not exceptional. Some enzymes, such as adenosine deaminase and phosphorylase, remain active for days *post mortem*, whereas other enzymes, such as LDH, start to loose activity within few hours *post mortem* (Bodwell et al., 1965; Tsai et al., 1972; Fischer et al., 1979). Furthermore, the RN^- mutation is located in the gene coding for adenosine monophosphate-activated protein kinase (AMPK), which is a key metabolic enzyme (Milan et al., 2000). Activated AMPK inhibits the ATP-consuming pathways and stimulates ATP-generating pathways (Hardie & Carling, 1997). Moreover, it has recently been reported by Shen and Du (2005) that AMPK is important for maintaining the phosphorylase activity in the muscle *post mortem*. Thus the mutation in the AMPK gene *per se*, and not the high glycogen content, might be an alternative explanation for the prolonged high GDE activity that was observed in the present study in RN^- carriers.

In summary, the *post mortem* GDE activity remains high for several hours both in RN^- carrier pigs and in wild type pigs. From 24 to 48 h *post mortem*, the GDE activity was significantly higher in RN^- carriers than in the wild type pigs. Thus, the differences in the GDE activity between the genotypes do not explain the fast pH decrease in RN^- carriers early

post mortem. However, the longer period of high GDE activity in RN^- carriers may enable the extended pH decrease.

7 CONCLUSIONS

This study provides new knowledge on the glycogen debranching enzyme (GDE) activity in the muscles of meat production animals and its role in degradation of glycogen and *post mortem* pH decrease. The present study showed that:

- The GDE activity is: high in porcine muscles, intermediate in bovine muscles and low in avian muscles. The phosphorylase (*a*+*b*) activity is high in chicken muscles compared to pig and cattle.
- In pigs and cattle, GDE and phosphorylase (*a*+*b*) activities increase with the increasing fast twitch and glycolytic character of a muscle in any individual animal. The high activities of these enzymes enable a fast rate of glycogenolysis. In the chicken, there is an increase in the phosphorylase activity, but a decrease in GDE activity in fast twitch glycolytic muscle compared to those found in slow twitch oxidative muscles.
- The calculated phosphorylase (*a*+*b*) and GDE activity ratios are relatively higher in fast twitch glycolytic muscles than in slow twitch oxidative muscles. This indicates that especially in fast twitch glycolytic muscles, the GDE may restrict the rate of glycolysis.
- The decrease in the GDE activity in the porcine *M. longissimus dorsi post mortem* is slow, and only becomes significant after several hours *post mortem*. In RN^- carriers with high muscle glycogen contents the decrease in GDE activity with time *post mortem* was even slower than in wild type pigs in which the muscle glycogen content was lower. The prolongation of high GDE activity may be one factor that enables the extended pH decrease in RN^- carriers.
- Temperature decrease *post mortem* strongly inhibits GDE activity in porcine muscles, but an increase in temperature to a few degrees above normal body temperature has no significant effect on GDE activity.
- The effect of temperature decrease became significant at higher temperatures in slow twitch oxidative muscles compared with fast twitch glycolytic muscles.
- The change in pH from values prevailing in living muscle to ultimate pH value in meat does not have a significant effect on porcine muscle GDE activity.
- Under normal conditions, the GDE is not the primary factor determining the rate or the extent of *post mortem* glycogenolysis in meat. However under certain conditions, such as in very fast chilling, the decrease in GDE activity of meat may reduce the rate of pH decrease and result in higher ultimate pH. Furthermore, the amount of glycogen and the length of its constituent A-chains determine when the GDE activity is needed.

8 SUGGESTIONS FOR FURTHER STUDIES

The studies in the present thesis have shown that there are differences in GDE activity between animal species and between different muscles of an animal. *Post mortem* changes in meat, particularly the temperature decline, significantly decrease GDE activity, which may have an influence on the rate and extent of the decrease in pH. Furthermore, the GDE may have an important role in restraining the rate of glycolysis in living muscles. Future studies should focus on the following:

- The activity of GDE is relevant when the length of the A-chain in glycogen is four glucose units. The length of the A-chains of muscle glycogen at death and its relationship to the rate of *post mortem* pH decrease warrants further study.
- The relationship of GDE activity to the rate and also the extent of the decrease in pH in muscles where glycogen content prior to slaughter is low and in muscles with high glycogen content also warrant further investigation.
- Salt-curing of *pre rigor* meat leads to higher ultimate pH values than in untreated meat. The effect of salt on the activity of GDE is an interesting question.
- It seems that the GDE activity might be related to the attainment of ultimate pH. Therefore it remains to be investigated if a difference in GDE activity late *post mortem* between the RN genotypes would lead to differences in the ultimate pH of the *M. semimembranosus* as was the case for the *M. longissimus dorsi*.
- The eventual impact of GDE activity on PSE pork and on cold shortening in beef.
- In RN^- carrier pigs, an increase in macro-glycogen content was observed at 96 h *post mortem*. The change might be due to an increase in the amounts of G-6-P and free glucose during the *post mortem* period, but this needs to be verified.
- In chicken muscles the ultimate pH value is generally higher than in red meat species. The buffering effect of *post mortem* phosphocreatine breakdown in chicken muscles is worth studying.

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APPENDIX A (ORIGINAL PAPERS I-IV)